Development of Novel Conjugate Vaccines Against *Salmonella*

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Abbreviations

2AB	2-aminobenzamide
[^{3}H]	Tritium
ABC	ATP-binding cassette
CDP	cytidine diphosphate
CFU	colony forming unit
CID	collisionally induced dissociation
CPS	capsular polysaccharide
CTAB	hexadecyltrimethylammonium bromide
CV	column volume
Dol-P	dolichyl phosphate
Dol-PP	dolichyl pyrophosphate
ECA	enterobacterial common antigen
ELISA	enzyme-linked immunosorbent assay
EPA	exotoxin A of *Pseudomonas aeruginosa*
EPI	Expanded Program on Immunization
GalNAcA	N-acetylgalactosaminuronate
GalNAcAN	N-acetylgalactosaminuronamide
GlcNAc	N-acetylglucosamine
HPLC	high-performance liquid chromatography
Ig	immunoglobulin
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kdo	2-Keto-3-deoxy-D-mannooctonic acid
LLO	lipid-linked oligosaccharide
LPS	lipopolysaccharide
MALDI	matrix-assisted laser desorption/ionization
MDR	multi-drug resistant
MS	mass spectrometry
m/z	mass-to-charge ratio
NTS	nontyphoidal *Salmonella*
OD	optical density
OPX	outer membrane polysaccharide export
O-SP	O specific polysaccharide (O antigen)

OST	oligosaccharyltransferase
PAMPs	pathogen-associated molecular patterns
PBS	phasphate buffered saline
PCP	polysaccharide copolymerase
RT	room temperature
RU	repeating unit
SCV	*Salmonella* containing vacuole
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sn	supernatant
SPI	*Salmonella* pathogenicity island
TEM	transmission electron microscopy
TTSS	type three secretion system
UDP	uridine diphosphate
Und-P	undecaprenyl phosphate
Und-PP	undecaprenyl pyrophosphate
VAERS	Vaccine Adverse Event Reporting System
Wc	whole cell
WHO	World Health Organization

Abstract

Various *Salmonella enterica* serovars can cause human diseases that range in severity from a mild gastroenteritis to a severe systemic infection known as typhoid fever. The emergence of antibiotic resistance in *S. enterica* strains poses a major problem in treatment of patients in many regions of the world. Currently, two licensed vaccines exist, conferring protection against *S. enterica* serovar Typhi, the causative agent of human typhoid. Unfortunately, these typhoid vaccines are only moderately immunogenic in infants and young children and revaccination is required every few years. Therefore, new vaccines are needed, which not only confer long-term protection against *S.* Typhi but also other harmful *S. enterica* serovars.

Conjugate vaccines are among the most effective and safe vaccines against bacterial diseases and have been used in humans for over 30 years. They are composed of an antigenic cell surface polysaccharide, purified from the pathogen, chemically coupled to a carrier protein. Several clinical trials have shown that glycoconjugates are promising vaccine candidates to prevent *Salmonella* infections. However, manufacturing of conjugate vaccines is a complex, multi-step process. An alternative approach to produce glycoconjugates is based on the bacterial N-linked protein glycosylation system first described in *Campylobacter jejuni*. This protein modification system was functionally transferred into *E. coli*, enabling production of customized recombinant glycoproteins *in vivo*. In this dissertation, the possibilities of using the *in vivo* conjugation technology for production of conjugate vaccines against various *S. enterica* serovars causing human diseases were explored.

Chemical coupling of the purified capsular polysaccharide of *S.* Typhi, known as Vi antigen, to a protein carrier resulted in a vaccine candidate that can be used for administration to infants as part of their routine immunization in endemic areas. Several key aspects of Vi capsule biosynthesis and its accessibility to the

bacterial oligosaccharyltransferase PglB, the key enzyme forming the N-glycosidic linkage, remain unresolved. Therefore, a molecular characterization of the *viaB* locus, encoding the biosynthetic machinery for Vi capsule formation, has been conducted. The study provides a comprehensive phenotypic analysis of single gene transposon insertion mutants using diverse microscopic and biochemical techniques. There were no indications that the Vi antigen is assembled on the lipid carrier undecaprenyl pyrophosphate (Und-PP) and no periplasmic intermediates exist, which are both prerequisites for being a substrate of PglB. Therefore, polysaccharide structures that fulfill these requirements and mimic the Vi were evaluated for their potential use as a typhoid vaccine component. The *E. coli* O121 O antigen cluster was genetically engineered, resulting in expression of an O specific polysaccharide reactive with antibodies raised against the Vi antigen. The structure of the recombinantly expressed O antigen was elucidated using a novel HPLC and mass spectrometry based method for purified Und-PP-linked glycans. Glycoconjugates were produced using the bacterial N-glycosylation system and their immunogenicity was evaluated in mice.

Expression of the Vi capsule is largely restricted to *S.* Typhi. Other harmful *S. enterica* serovars express O antigens, which have been used as components of conjugate vaccine candidates against *S.* Paratyphi A or *S.* Typhimurium. These O specific polysaccharides have a galactose at the reducing end in common. It has been reported that such glycan structures do not serve as substrates for PglB, because the reducing end saccharide should contain an acetamido group at C-2, thought to be essentially involved in catalysis. Nonetheless, it was shown in this work that the O antigen of *S.* Typhimurium is transferred to acceptor proteins upon optimization of recombinant PglB expression. This finding opens up the possibility of producing novel conjugate vaccines against *Salmonella*.

Zusammenfassung

Infektionen mit Salmonellen können beim Menschen zu unterschiedlich schweren Krankheitsbildern führen die von einer ungefährlichen Gastroenteritis bis zu lebensbedrohendem Typhus reichen. Die Verbreitung von Antibiotika-resistenten Salmonellen Stämmen erschwert die Behandlung von Patienten massgeblich. Es existieren zwei Impfstoffe die gegen eine Infektion mit dem Typhuserreger, *Salmonella enterica* Serovar Typhi, schützen. Diese Impfstoffe haben aber den Nachteil, dass sie bei Kleinkindern unwirksam sind und der Impfschutz alle paar Jahre erneuert werden muss. Demzufolge werden neue Impfstoffe gebraucht, die einen langzeitigen Schutz nicht nur gegen den Typhuserreger sondern auch andere gesundheitsgefährdende Salmonellen Serovare vermitteln.

Konjugierte Impfstoffe gehören zu den wirksamsten und sichersten vorbeugenden Massnahmen gegen bakterielle Erreger. Sie entstehen in einem aufwendigen chemischen Prozess, bei dem Antigene in Form von Polysacchariden, die vom Krankheitserreger isoliert werden, an Trägerproteine gekoppelt werden. Mehrere klinische Studien haben gezeigt, dass solche Glykoproteine vielversprechende Impfstoffe darstellen, die wirksam Salmonellen Infektionen vorbeugen können. Eine elegantere Herstellungsmethode beruht auf der bakteriellen Biosynthese von N-glykosidisch gebundenen Glykoproteinen, die zum ersten Mal in *Campylobacter jejuni* beschrieben wurde. Es wurde gezeigt, dass *E. coli* Zellen derart genetisch verändert werden können, dass sie massgefertigte N-glykosidisch gebundene Glykoproteine *in vivo* produzieren. In dieser Dissertation wurde diese neuartige Herstellungsmethode zur Entwicklung von konjugierten Impfstoffen gegen Salmonellen angewandt.

Ein konjugiertes Vakzin, das durch die chemische Kopplung des kapsulären Polysaccharids des Typhuserregers (Vi Antigen) an ein Trägerprotein hergestellt wird, wurde bereits erfolgreich

in Kleinkindern getestet und vermittelt einen wirksamen Schutz gegen Typhus. Es ist jedoch fraglich, ob das Vi Antigen ein Substrat der Oligosaccharyltransferase PglB, dem Schlüsselenzym der bakteriellen N-Glykosilierung darstellt. Verschiedene Aspekte der Vi Biosynthese sich noch immer unklar. Deshalb wurde eine molekulare Charakterisierung des *viaB* Lokus durchgeführt, welcher die biochemische Maschinerie zur Vi Kapsel Expression kodiert. Es wurden keine Anzeichen dafür gefunden, dass das Vi Antigen auf dem Trägerlipid Undecaprenyl-Pyrophosphat (Und-PP) aufgebaut wird. Des Weiteren wurden keine periplasmatischen Zwischenprodukte beobachtet. Dies wären aber beides Voraussetzungen an ein Substrat der bakteriellen Oligosaccharyltransferase. In Bakterien existiert aber eine enorme Vielfalt unterschiedlichster Zuckerstrukturen. Einige ähneln in ihrer chemischen Struktur dem Vi Antigen und erfüllen auch die Anforderungen eines Substrats von PglB. So wurde der O Antigen Lokus von *Escherichia coli* O121 genetisch modifiziert, dass die Zellen ein Polysaccharid exprimierten, das von Vi spezifischen Antiköpern erkannt wird. Mit Hilfe einer neuartigen Methode zur Strukturaufklärung von Und-PP gebundenen Oligosacchariden wurde die Ähnlichkeit dieses veränderten O Antigens zum Vi Antigen nachgewiesen. Nachdem Glykoproteine mit Hilfe von genetisch Veränderten *E. coli* Zellen hergestellt wurden, wurde deren Immunogenität in Mäusen evaluiert.

Die Expression des Vi Antigens ist aber beschränkt auf wenige Salmonellen Serovare, darunter *S.* Typhi. Andere gesundheitsgefährdende Salmonellen Stämme exprimieren O Antigene, welche ebenfalls als Bestandteil von konjugierten Impfstoffen gegen *S.* Paratyphi A oder *S.* Typhimurium erfolgreich evaluiert wurden. Die Polysaccharidstrukturen dieser sehr ähnlichen O Antigene besitzen alle eine Galaktose am reduzierenden Ende. Es wurde berichtet, dass diese Zuckerstrukturen nicht durch PglB auf Akzeptorproteine übertragen werden können, da dem Zuckermolekül des reduzierenden Endes eine N-Acetylgruppe am C-2 fehlt, welche vermutlich essentiell im katalytischen Mechanismus involviert ist. In dieser Arbeit konnte gezeigt werden, dass diese

Polysaccharide dennoch durch PglB übertragen werden können. Dies eröffnet die Möglichkeit, neuartige konjugierte Impfstoffe gegen diverse krankheitsrelevante Salmonellen Serovare herzustellen.

Introduction

Nomenclature and taxonomy of the genus *Salmonella*

Salmonella are enteric, Gram-negative, facultative anaerobic, rod-shaped bacilli that are a major cause of infectious disease worldwide. *Salmonella* is a genus of the large family of *enterobacteriaceae* that includes other well-known pathogens like *Escherichia*, *Yersinia*, *Shigella* and *Klebsiella*. The taxonomy within the genus *Salmonella* continues to evolve and is a source of considerable confusion.

Salmonella is named after an American bacteriologist, D. E. Salmon, who identified the causative agent of swine fever in 1885 (*Salmonella choleraesuis*). Originally, the species within the genus *Salmonella* were defined according to the disease they elicit (*Salmonella typhimurium*: typhoid fever in mice; *Salmonella abortusovis*: abort of sheep), or the geographical area where the organism was first isolated (*Salmonella dublin*, *Salmonella kentucky*). In 1966 F. Kauffman proposed that based on serologic classification determined using specific antibodies, each serovar should be considered as a separate species [1]. The most recent classification based on DNA relatedness and molecular analysis divides the genus *Salmonella* into two species: *Salmonella enterica* and *Salmonella bongori*, which are further subdivided into subspecies (ssp.) designated with Roman numerals. *Salmonella enterica* (*S. enterica*) consists of six subspecies: I, *S. enterica* ssp. *enterica*; II, *S. enterica* ssp.*salamae*; IIIa, *S. enterica* ssp.*arizonae*, IIIb; *S. enterica* ssp. *diarizonae*; IV, *S. enterica* ssp. *houtenae*; and VI, *S. enterica* ssp. *indica*. Subspecies V represents the species *Salmonella bongori*. The subspecies are again further subdivided into serovars (serotypes) and strains **(Figure 1)**. Subspecies I of *Salmonella enterica* comprises the medically important serovars and accounts for 99.5% of isolated *Salmonella* strains. These serotypes are still referred to by their previous genus or species designation. For example, *Salmonella enterica* ssp. *en-*

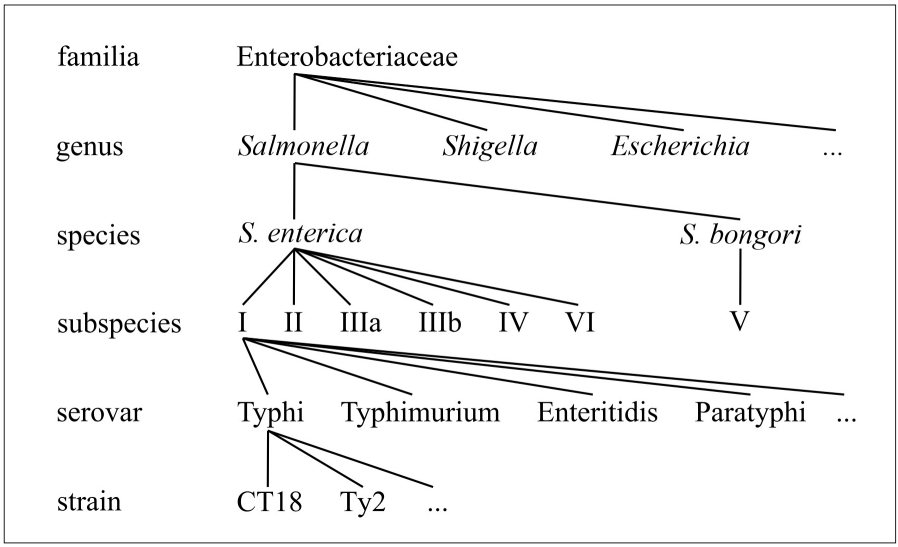


Figure 1: Taxonomy of the genus *Salmonella*.
Salmonella is a genus of the family of *enterobacteriaceae*. The genus *Salmonella* is divided into two species: *Salmonella enterica* and *Salmonella bongori.* These species are further subdivided into subspecies, serovars (serotypes) and finally strains. Subspecies I of *Salmonella enterica* comprises the medically important serovars, *e.g. Salmonella enterica* subspecies I serovar Typhi (*S.* Typhi).

terica serovar Typhi continues to be referred as *Salmonella typhi* or *Salmonella* Typhi whereas serovars of other subspecies are designated by their antigenic formula. In this work the terminology *Salmonella* Typhi (*S.* Typhi) is used.

The serovar classification system is based on the diversity of antigens present on the cell surface that can be analyzed in agglutination tests using specific antibodies. In *Salmonella* three different cell surface antigens are examined: The H, O and Vi antigen **(Figure 2)**.

Almost all *Salmonella* serovars are motile, and express peritrichous flagella that bear flagellar (H) antigens. H is derived from the German word "Hauch" (breath) and refers to the colony mor-

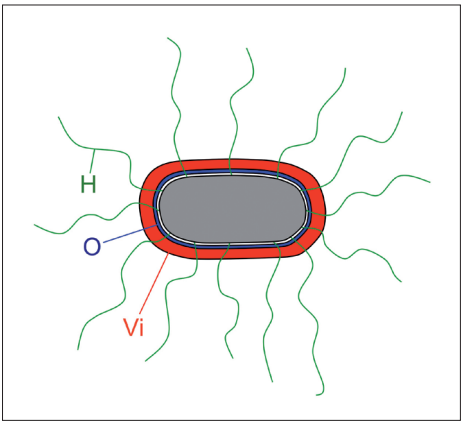


Figure 2. Bacterial cell surface antigens.
The serovar classification system in *Salmonella* is based on the examination of three different cell surface antigens: the H, O, and Vi antigen. The H antigen refers to the peritrichous flagella expressed by *Salmonella*. The O antigen is part of the lipopolysaccharide (LPS), found in the outer membrane of Gram-negative bacteria. Certain *Salmonella* serovars express a capsular polysaccharide known as the Vi antigen. The presence of the Vi capsule masks the subjacent O antigen and Vi positive *Salmonella* strains do not agglutinate with O specific antibodies.

phology of motile bacteria on semisolid agar, which reminds one of the image that is generated by breathing on a glass surface. Most serotypes are biphasic, *i.e.* their genomes encode two different forms of flagellar antigen whereof one is expressed on the cell surface, but with a low frequency the cell can switch to the expression of the other phase. In serovars capable of phase shifting both phases have to be determined in the classification process.

O antigens are part of the lipopolysaccharide (LPS), found in the outer membrane of Gram-negative bacteria. This antigen was first discovered on non-motile bacteria. Therefore, the O refers to the German term "ohne Hauch" (without breath). The LPS consists of a conserved lipid part, the lipid A, the core oligosaccharide and a highly variable repetitive glycan polymer, referred

to as the O antigen or O specific polysaccharide (see page 45*ff.* about the biosynthesis of *Salmonlla* O antigens).

The capsular polysaccharide expressed by a few *Salmonella* serovars is known as the Vi antigen. The Vi was first associated with virulence because it is expressed by the important human pathogen *S.* Typhi, the causative agent of typhoid fever. The presence of the Vi capsule masks the subjacent O antigen and inhibits reaction with O specific antibodies.

The antigenic formulae of *Salmonella* serovars are listed in a document called the White-Kauffmann-Le Minor scheme [2]. Today, more than 2500 different *Salmonella* serovars have been described. The vast variety of antigens found on the cell surface of the pathogen possibly indicates that diversification was driven by immune selection.

Evolution and host adaption of *Salmonella* serovars

Different bacterial isolates of the genus *Salmonella* not only show a great variability of cell surface antigens but also differ in terms of host adaption and virulence potential. During evolution *Salmonella* spp. have adapted to colonize many different niches and hosts. They can be found as commensals and pathogens in a wide range of cold and warm-blooded animals. Bäumler *et al.* wrote: "Studying the evolution of host adaption and understanding the strategies, which a pathogen develops to resist the host's immune system, is also fundamental in understanding both the origin of infectious disease and the emergence of new pathogens" [3].

It is estimated that the genus *Salmonella* has diverged from *Escherichia coli* around 100 million years ago. During the evolution of virulence *Salmonella* spp. have acquired several large genomic insertions by horizontal gene transfer. These insertions, also known as *Salmonella* pathogenicity islands (SPI), encode genes important for virulence and survival in the host. SPI possess a G+C content, which differs from that of the genomic backbone and they are of-

ten integrated at tRNA gene sites. The acquirement of complex virulence modules by horizontal gene transfer dramatically accelerates the evolution of pathogenicity and it is referred to as evolution in quantum leaps [4]. It was postulated that the virulence in the genus *Salmonella* evolved in three phases [3]:

1 The first phase involved the acquisition of the *Salmonella* pathogenicity island 1 (SPI1) by horizontal gene transfer. SPI1 is found in all phylogenetic lineages of the genus *Salmonella* but is absent from the chromosome of *Escherichia coli*. SPI1 encodes a type-III secretion system (TTSS), which is a complex, needle-like molecular apparatus that is structurally related to the bacterial flagellum. By means of the TTSS, bacteria can directly inject a cocktail of virulence proteins (effectors) from the bacterial cell cytoplasm into the host cell. In the host cell cytoplasm the effectors interfere and modulate signaling pathways. The SPI1 encoded TTSS is used during the intestinal phase of infection and mediates invasion into non-phagocytic cells, triggering of proinflammatory cytokine production and induction of cell death of host phagocytes [5,6].

2 The second phase of evolution represents the formation of the two *Salmonella* species *Salmonella enterica* and *Salmonella bongori*. The divergence of the *Salmonella* lineages is characterized by the acquisition of the *Salmonella* pathogenicity island 2 (SPI2), which is only found in the chromosome of *Salmonella enterica*. The SPI2 encodes a second TTSS, which is used during the systemic phase of infection and the colonization of deeper tissues. Recently it has been shown, that the SPI2 encoded TTSS is involved in pathogen traffic to the basolateral side and exit into the lamina propria once the bacteria has invaded epithelial cells [7].

3 The differentiation of *Salmonella enterica* into the distinct phylogenetic subspecies reflects the third phase of virulence evolution. This phase is characterized by a dramatic expansion in

host range. Whereas *Salmonella bongori* and *Salmonella enterica* subspecies II, IIIa, IIIb, IV, VI are mainly associated with cold-blooded vertebrates, *S. enterica* ssp. I expanded its host range to warm-blooded vertebrates.

A new barrier encountered by *S. enterica* ssp. I serovars was the immune system of higher vertebrates, which is higher developed and comprises peripheral lymphoid filter organs, the lymph nodes. New strategies had to be developed by the pathogen to adapt to these systems of host defence.

Distinct serovars of *S. enterica* ssp. I are differently well adapted to a specific host. For example, *S.* Typhi is only isolated from humans, whereas *S.* Typhimurium is found to have a broad host range and is associated with infections not only in humans but many other warm-blooded vertebrates. In most cases, the human immune system can successfully limit bacterial expansion of *S.* Typhimurium to the intestine and the gut associated lymphoid system. In contrast, the human restricted serovar *S.* Typhi causes the systemic infection, known as typhoid fever. Furthermore, host specific serovars like *S.* Typhi have acquired the ability to infect full-grown animals, whereas broad host range serovars tend to be more frequently associated with disease in young animals, indicating that they are not fully adapted to the fully mature immune system. Additionally, host specific serovars tend to be more virulent. For example, typhoid fever results in mortality rates of 10–20% in the preantibiotic era, whereas human illness caused by the broad host range serovar *S.* Typhimurium or *S.* Enteritidis causes death in only 0.5% of the cases [8]. It has been proposed that pathogens causing high mortality rates are not well adapted to their hosts, because they destroy their habitats and reduce their transmissibility. Less virulent forms should therefore be preferably selected during evolution. However, the opposite has been observed for the evolution of *S.* Typhi and the question arose what selective forces were responsible for the increase of virulence that accompanied the adaption of *S.* Typhi to humans [3]. It has been noticed that patients recovering from typhoid fever more frequently become

chronic carriers as compared to patients with nontyphoidal Salmonellosis. A chronic, asymptomatic carrier has the potential to infect many other individuals, thereby increasing the transmissibility of the pathogen. Therefore, this mechanism seems to represent the evolutionary pressure on development towards a higher degree of virulence.

The host restriction of *S.* Typhi might have evolved through loss of gene function. The *S.* Typhi genome possesses around 204 pseudogenes, *i. e.* genes that have been inactivated by mutations [9], including several that play a role in virulence in *S.* Typhimurium. In the broad host range pathogen *S.* Typhimurium only 39 pseudogenes are found. The inactivation of genes responsible for host interaction might explain the host restriction of *S.* Typhi, which is not seen in other serovars.

***Salmonella* infections: pathogenesis and disease**

Salmonella infections are an important public health problem worldwide and it has been estimated that this pathogen is responsible for 3 billion human infections each year. It is important to note that infection by different *Salmonella* serotypes can result in diverse clinical syndromes. The disease manifestation elicited upon interaction between the pathogen and the host is not only dependent on the serotype, but also on the host species, infecting dose, immunologic competence and the gut flora. In humans, the disease pattern caused by *Salmonella* infections can be divided into typhoid fever (enteric fever) and non-typhoidal salmonellosis (NTS). Typhoid fever is a systemic infection, most commonly caused by the human restricted serovars *S.* Typhi and *S.* Paratyphi. On the other hand, non-typhoidal salmonellosis is a gut-associated, self-limiting gastroenteritis, which in developed countries is mostly caused by *S.* Typhimurium and *S.* Enteritidis [10]. Non-typhoidal *Salmonella* serovars have a broad host range and are frequently zoonotic. However, NTS serovars have emerged as a major cause

of invasive bacterial bloodstream infections in sub-Saharan Africa, amongst children with malaria and malnutrition, and amongst HIV-infected adults (reviewed in [11]). *S.* Typhi and *S.* Paratyphi colonize only in humans and infections are mostly acquired from water or food contaminated with human feces. In the following sections the pathogenesis of *Salmonella* infection and the resulting human diseases are described.

Pathogenesis

A general characteristic of *Salmonella* serovars is the ability to invade and persist in host cells. They are well adapted to an intracellular lifestyle and can even survive in macrophages and other immune cells.

Susceptible hosts typically ingest the pathogen orally in contaminated food and water. The dose of ingested bacilli greatly influences the onset of the disease. Volunteer studies with *S.* Typhi showed that subjects who ingested 10^3 organisms did not develop illness whereas doses of 10^5 and 10^9 caused typhoid fever in 28–55 % and 98 % of the volunteers respectively [12]. The low pH environment of the stomach, which the ingested organisms encounter first, represents an initial barrier to infection. Conditions that increase the gastric pH, decrease the infectious dose [13]. However, *Salmonella* have an adaptive acid tolerance response, which enables the organism to withstand acidic conditions also found in the phagosomal vacuole encountered in a later step of pathogenesis [14].

The epithelial barrier in the intestine forms the next barrier that is met by *Salmonella* during infection. The bacteria can breach the epithelial barrier by different mechanisms to arrive in the lamina propria. *Salmonella* are regarded as invasive bacteria and as mentioned on page 16*ff.* , *Salmonella enterica* subspecies possess two Type III secretion systems encoded in SPI-1 and SPI-2 (TTSS-1, TTSS-2). The TTSS is a complex, needle-like molecular apparatus, which enables the bacterium to inject a cocktail of virulence proteins (effectors) into the host cell [15]. In the host cell these ef-

fector proteins interfere and modulate signalling pathways inducing amongst other things profound cytoskeletal rearrangements. This cytoskeletal remodeling drives localized membrane ruffles, that reach out and enclose adherent bacteria, triggering their internalization into membrane bound vacuoles [16]. The SPI-1 encoded TTSS and effector proteins are essential for invasion into non-phagocytic cells. Besides triggering their internalization, *Salmonella* spp. can also be actively taken up by lamina propria phagocytes or by microfold cells (M cells). The former were shown to protrude thin, arm-like extensions across the epithelium into the intestinal lumen thereby establishing another route of entry. M cells are specialized epithelial cells that sample intestinal antigens and transport these antigens to lymphoid cells in the underlining Peyer's patches. After entering M cells *Salmonella* can manipulate signalling pathways and induce for example apoptosis [17]. After the crossing of the epithelial M cells, *Salmonella* can access lymphoid cells. An additional invasion mechanism might involve paracellular traffic through the manipulation of tight junctions [18]. In a natural infection it is likely that *Salmonella* exploits a combination of different invasion strategies.

It remains equivocal, which host cell functions are hijacked by the pathogen, resulting in transcellular migration and exit into the lamina propria. However, it has recently been shown in the mouse model for *S.* Typhimurium-induced enterocolitis, that the pathogen requires the type-III secretion system encoded on SPI-2 (TTSS-2) for transepithelial traffic to the basolateral site of the cell where the pathogen is released by exocytosis [7].

Once the pathogen has reached the lamina propria *Salmonella* encounter another obstacle of innate immunity, the submucosal phagocytes. In addition to the invasion of non-phagocytic cells, the ability to survive and replicate in host cells represents a further feature of *Salmonella* pathogenesis. The TTSS-2 and its effectors are fundamental to the intracellular lifestyle. In contrast to other facultative intracellular pathogens like *Shigella* spp. and *Listeria monocytogenes*, *Salmonella* spp. do not enter the cytoplasm but remain in the phagosomal vacuole. They can inhibit the maturation

of the phagosome, thereby forming a *Salmonella* containing vacuole (SCV). By this mechanism the pathogen generates a protected niche where it can survive and replicate.

In addition to invasion of the intestinal epithelial cell layer, non-typhoidal *Salmonella* serotypes induce a local inflammatory response, provoking recruitment and transmigration of polymorphonuclear leukocytes, including neutrophils, to the intestinal lumen and diarrhea. The pathogen exploits inflammation to compete with the intestinal microbiota and overcome colonization resistance [19]. However, infections with the human restricted serotypes *S.* Typhi or *S.* Paratyphi result in a systemic spread of the pathogen. Little is known about the mechanism that allows typhoidal *Salmonella* serotypes to become systemic whereas NTS are mostly restricted to the intestine in immunocompetent individuals. It seems that *S.* Typhi remains relatively undetected by the immune system during the invasion of the tissue, which is also characterized by the general lack of polymorphonuclear leukocyte influx. There is some evidence that the capsular polysaccharide expressed by *S.* Typhi, the Vi antigen, has anti-inflammatory properties by masking pathogen-associated molecular patterns (PAMPs) and by suppressing early inflammatory responses in intestinal epithelial cells [20,21]. The survival of *S.* Typhi in macrophages is essential for efficient systemic infection. Migration of infected phagocytes to other organs of the reticuloendothelial system facilitates dissemination of bacteria in the host. *S.* Typhi resides within cells throughout the organs of the reticuloendothelial system during an incubation period of 8–14 days. After that period the bacteria are released from this sequestered intracellular habitat and the onset of clinical typhoid fever is accompanied by a sustained bacteremia. In the bacteremic phase, the organism is widely disseminated.

Another important characteristic of *S.* Typhi is the predilection of the pathogen to reach the gallbladder. Gallbladder infection becomes chronic in up to 6% of infected individuals [22]. *S.* Typhi persists in these healthy carriers over long periods in the absence of clinical symptoms. These asymptomatic *S.* Typhi carriers are a key reservoir of typhoid infections.

Typhoid fever

Causative agent and Epidemiology

Typhoid fever remains a serious public health problem of which there are 22–33 million cases occurring each year, including about 216'000–500'000 deaths [23]. However, it is difficult to quantify the number of typhoid fever incidences, because the clinical symptoms can be confused with other febrile illnesses and detailed bacteriological and serological diagnosis are often not available in endemic areas. The causative agent of this human systemic infection is *S.* Typhi. *S.* Paratyphi, mainly *S.* Paratyphi A or B (infections with *S.* Paratyphi C are rare), cause paratyphoid fever, which is clinically indistinguishable from typhoid fever. Typhoid and paratyphoid fevers are referred to as enteric fevers. Typhoid fever accounts for > 75 % of the clinical cases in endemic regions whereas paratyphoid fever accounts for the rest. However, in certain regions where typhoid vaccination programs are ongoing a decline in *S.* Typhi incidences was observed with a concomitant rise in *S.* Paratyphi infections.

S. Typhi and *S.* Paratyphi express several cell surface antigens. Besides the flagellar (H) antigen and the lipopolysaccharide (LPS) O antigen *S.* Typhi and *S.* Paratyphi C also express a capsular polysaccharide known as the Vi antigen. The presence of the Vi antigen prevents binding of O specific antibodies to the O antigen.

S. Typhi and *S.* Paratyphi are human restricted pathogens and therefore humans, *i.e.* short term and chronic carriers, are the sole reservoir. The infection is orally transmitted through water and food that has been contaminated with human fecal material. The occurrence of typhoid fever correlates to the availability of sewage treatment infrastructure and the quality of water supply. Hence incidences of typhoid fever drastically dropped in Europe and the United States in the 20th century with the introduction of water treatment. Today, typhoid fever remains endemic in less-developed countries, where sanitary conditions remain poor. This includes many countries in Africa, Asia, and South America.

In most endemic areas, the peak incidence of typhoid fever is typically observed in schoolchildren and young adults (5–19 years). In children younger than 2 years of age, infections with *S.* Typhi are often unrecognized because of atypical clinical symptoms and difficulties of confirmation of the diagnosis due to the volume of blood that can be drawn for culture. However, in newer surveys using active surveillance methods a high incidence of typhoid fever was observed in young children two to four years of age [24,25].

Clinical course of typhoid fever

The clinical course and severity of typhoid fever varies greatly between different patients. Important factors that determine the clinical manifestations of the disease are the age and the immunologic competence of the patient. Additionally it seems important whether the patient has grown up in an industrialized country or in a country where typhoid fever is endemic. Travelers or military personnel from industrialized countries who visit endemic areas are especially at risk. These groups lack a certain background immunity, which the indigenous population acquired through constant contact with the pathogen, often in doses that were not sufficient for developing typhoid fever.

The onset of the disease, which corresponds to the bacteremic phase, begins typically after an incubation time of 8–14 days. Classic cases begin with influenza-like symptoms with malaise, chills, anorexia, myalgia, abdominal discomfort, headache and fever that increases to reach 39–41 °C. A dry cough is also common in the early stages of illness. Adults often have constipation whereas diarrhea may occur in young children. Abdominal symptoms are usually rare in patients. Initially, the fever is low but rises in a stepwise manner by daily increments of 0.5–1 °C. By the second week a sustained fever of 39–41 °C is present. Without any treatment the fever remains at this level for 10–14 days. During the period of high fever, 5–30 % of the patients manifest rose spots, which are skin lesions approximately 2–4 mm in diameter and localized on the abdomen and chest and more rarely on the back, legs and

arms. *S.* Typhi can be cultured from rose spots [26]. Occasionally, cerebral dysfunction including delirium, coma and shock are observed in severe cases of typhoid fever. With convalescence, the fever decreases also in a stepwise fashion.

Complications occur often in patients that have been ill for more than two weeks. The most feared complications are gastrointestinal bleeding, intestinal perforation and typhoid encephalopathy. In up to 6% of patients with typhoid fever the gallbladder infection becomes chronic. These individuals are asymptomatic carriers and therefore an important reservoir of the human restricted pathogen.

Immunity that follows clinical infection with pathogenic *S.* Typhi is relative and can be overcome [27]. Volunteer studies have shown that prior infection with *S.* Typhi induced immunity that modestly protected against subsequent challenges with virulent bacteria. A study carried out by DuPont *et al.* [28] showed that the protective effect of a prior infection was only 33%.

Treatment and emergence of antibiotic resistant strains

Before the discovery of antibiotics, typhoid fever had a case-fatality rate of 10–20% and hence was a much-feared disease. In 1948 it was found that chloramphenicol can successfully be used to treat typhoid fever [29]. After the introduction of antibiotics the case fatality dropped to well below 1%. However, in the early 1970s antibiotic resistance emerged in *S.* Typhi and the treatment of typhoid fever with chloramphenicol became increasingly ineffective. Outbreaks with chloramphenicol resistant strains occurred in Mexico, India, Vietnam, Thailand, Korea, and Peru [30]. The resistance to chloramphenicol was often encoded in combination with resistance to streptomycin, sulfonamides, and tetracycline by a self-transferable plasmid of the H1 incompatibility group. At the beginning of the 1990s, *S.* Typhi strains emerged with resistance to all first line antimicrobials used at that time, like chloramphenicol, trimethoprim, sulfamethoxazole, and ampicillin. The resistant genes of these multi-drug resistant (MDR) strains were also en-

coded on the *Inc*H1 plasmid and MDR strains disseminated throughout Asia and northeast Africa.

In 1962, the quinolone derivative, nalidixic acid was discovered. The subgroup of fluoroquinolones, containing a fluorine atom attached to the quinolone framework, has a greater antimicrobial activity and represents the majority of quinolones used clinically. Quinolones and fluoroquinolones are a group of synthetic antibiotics that target the DNA gyrase and topoisomerase in bacteria and thereby inhibit the transcription and replication of the bacterial DNA. Today, fluoroquinolones (*e.g.* ciprofloxacin, ofloxacin, fleroxacin, pefloxacin) are the most efficient drugs used for the treatment of typhoid fever [31,32]. But the emergence of nalidixic acid resistant *S.* Typhi isolates and strains with reduced susceptibility to fluoroquinolones have become a major problem in Asia [32]. Resistance in *S.* Typhi is usually associated with single point mutations in the DNA gyrase gene *gyrA*, typically in the quinolone resistance-determining region.

Full fluoroquinolone resistant *S.* Typhi strains are still rare and other antibiotics like azithromycin or the cephalosporins are the drugs of choice to cure typhoid fever caused by these multidrug resistant strains. A drawback of these antibiotics is that they are relatively expensive and not readily available in rural areas of less developed countries. In areas where quinolone resistant strains are uncommon, fluoroquinolones are the favored antibiotics and inexpensive antibiotics, like chloramphenicol, amoxicillin, and trimethoprim-sulfamethoxazole are still used for the treatment of typhoid fever caused by *S.* Typhi strains fully susceptible to these antibiotics [32]. Fluoroquinolones are also the favored antibiotic to cure chronic carriers, *i.e.* patients with chronic gallbladder infections. Before this class of antibiotics was available, the infected gallbladder was surgically removed (cholecystectomy) [27].

The cost of the treatment is the critical factor in developing countries. The emergence of resistance in *S.* Typhi to widely used antibiotics and the necessity to use more expensive antimicrobials therefore poses a major problem in treatment of patients in many regions of the world.

Typhoid vaccines

The development of vaccines against typhoid fever was temporarily neglected because of the availability of inexpensive oral antibiotics. The emergence of *S.* Typhi strains with resistance to all oral antibiotics at the beginning of the 1990's led to an increase in typhoid mortality and also to an increased need for efficacious vaccines that can be included in broad immunization programs.

Regarding the pathogenesis of a *S.* Typhi infection, a protective role is probably played by secretory immunoglobulin A (IgA) (preventing the invasion of the intestinal epithelium), circulating antibodies (preventing the spreading during the bacteremic phase) and cell-mediated immunity (eliminating intracellular bacteria). Oral and parenteral vaccines have been developed that show significant protection by either eliciting a strong secretory intestinal IgA and cell-mediated immunity (oral route) or a predominant circulating antibody response (parenteral route). Until today there is no agreement on the critical antigens responsible for protection and variable cell surface structures might be exposed during the different phases of pathogenesis.

Populations that are at particularly high risk of developing typhoid fever would benefit from a vaccine. These include school-age children from countries where typhoid fever is endemic, travelers and military personnel from industrialized countries who are visiting endemic areas, and microbiology technicians in clinical laboratories working with *S.* Typhi.

Typhoid vaccines were already developed in 1896 in Germany and England [33,34]. These early vaccines consisted of killed and preserved *S.* Typhi bacilli that were usually injected subcutaneously (parenteral whole cell vaccines). Different methods of inactivating *S.* Typhi without destroying important cell surface epitopes have been tested. A heat-inactivated, phenol preserved whole cell parenteral vaccine is currently still licensed and was produced by Wyeth up through the 1990's for the U.S. Army. Because of frequent, severe adverse effects of this form of typhoid vaccine nearly all manufacturers stopped production. The other currently licensed, available and used vaccines are the purified Vi polysac-

charide parenteral vaccine and the attenuated strain Ty21a, which is used as a live oral vaccine. These two vaccines are more closely described in the following sections.

Purified Vi polysaccharide parenteral vaccine *S.* Typhi expresses a capsular polysaccharide known as Vi antigen (Vi). Vi is a linear, acidic homopolymer of α-1,4-linked *N*-acetylgalactosaminuronate (D-GalNAcA), variably O-acetylated at C-3 (biosynthesis of Vi is more closely described on page 43*f.*). The O-acetyl groups make up most of the surface and immunogenicity of Vi is closely related to the degree of O-acetylation [35,36]. In a modern purification procedure the polysaccharide is extracted from *S.* Typhi culture supernatant using hexadecyltrimethylammonium bromide, which preserves the important O- and N-acetyl groups. Earlier purification strategies resulted in partially de-acetylated and therefore less immunogenic Vi.

The purified Vi vaccine is produced by Sanofi Pasteur MSD (Typhim Vi®), GlaxoSmithKline (Typherix®) and several other manufacturers. The Vi vaccine is not protected by patent rights. Hence, the production technology can be easily transferred to manufacturers in countries where typhoid fever is endemic. These locally produced Vi vaccines are available at lower prices as compared to imported vaccines. An immunizing dose consists of 25 μg or 30 μg of Vi polysaccharide in 0.5 ml of isotonic buffer. The vaccine is usually administered as a single subcutaneous or intramuscular injection.

Besides the route of administration, number of doses required, vaccine stability is another important parameter. Especially for vaccines that are used in broad vaccination programs in the developing world, where the cold chain cannot be guaranteed. The purified Vi polysaccharide vaccine is highly stable and it retains its physicochemical characteristics even after 6 months' storage at 37 °C [27]. Nevertheless, manufacturers recommend that the vaccine is stored at 2–8 °C.

Clinical studies in several endemic and nonendemic countries have shown that the purified Vi vaccine elicits serum IgG Vi anti-

body responses in adults and children older than 2 years [37-42]. The usefulness of the Vi polysaccharide vaccine is limited by the fact that immune responses against polysaccharide antigens do not involve T-cells. Therefore, Vi is not a good immunogen in infants (< 2 years), immunological memory cannot be established, and revaccination does not elicit any booster effect [40,41].

Large field trials conducted in Nepal, South Africa and China with a single dose of 25 μg (30 μg in China) of Vi polysaccharide demonstrated a vaccine efficacy of 64–72% [37,38,43]. However, the duration of protective efficacy lasted only for 2–3 years [44], which implies that revaccination has to occur every 3 years.

Proteins involved in Vi capsule formation are encoded within a DNA island, termed *Salmonella* pathogenicity island 7 (SPI-7) [45]. Precise excision of the SPI-7 in Vi-negative mutants has been demonstrated, indicating that Vi antigen expression is located on a unstable region in the *S.* Typhi genome [46]. The presence of the Vi capsule is not essential for infection, as Vi-negative *S.* Typhi have been reported to cause outbreaks of typhoid fever [12,47-49]. It is unlikely that a Vi vaccine protects against Vi-negative *S.* Typhi and concerns have been raised, that the widespread use of the purified Vi polysaccharide vaccine might select for such strains.

The Vi vaccine is well tolerated and post-marketing surveillance from the Vaccine Adverse Event Reporting System (VAERS) of the USA reported an overall rate of 4.5 adverse events per 100'000 doses [50].

Ty21a live oral vaccine Ty21a is an attenuated *S.* Typhi strain used as a live oral vaccine and is derived from the wild-type strain Ty2 by nitrosoguanidine-induced mutagenesis. This mutant strain does not express the Vi antigen, shows no activity of the enzyme uridine diphosphate (UDP)-galactose-4-epimerase (GalE), and possesses a reduced activity of the enzymes galactokinase (GalK) and the galactose-1-phosphate uridyl transferase (GalT) [51]. These mutations in the galactose operon lead to a defect in sugar metabolism. Ty21a cannot convert UDP-glucose to UDP-galactose and vice versa due to the non-functional GalE. The nu-

cleotide activated galactose is the donor substrate for galactose incorporation into the outer core and the O antigen (described on page 45*ff.*) of the *S.* Typhi lipopolysaccharide (LPS). Therefore, when grown in the absence of galactose, Ty21a does not express the O antigen because no UDP-galactose is synthesized. In the presence of exogenous galactose, the galactokinase (GalK) and the galactose-1-phosphate uridyl transferase (GalT) convert galactose to UDP-galactose and the O antigen is assembled. Besides being incorporated into the LPS, UDP-galactose cannot be further metabolized because of the lack of the epimerase GalE. Therefore, Ty21a accumulates galactose-1-phosphate and UDP-galactose when grown in the presence of exogenous galactose, which again after reaching a critical concentration leads to cell instability and cell lysis [51,52]. In the production process of Ty21a, galactose is included in the medium to ensure that the O antigen is expressed which leads to a protective vaccine whereas the O antigen negative version is not [53].

However, the mutations in the galactose operon and the Vi-negative phenotype do not account for the attenuation of Ty21a. Construction of a Ty2 derivative containing defined deletions in the *galE* gene and the Vi biosynthetic gene cluster resulted in a strain that retained virulence in humans [47]. Two dozen additional mutations were identified in Ty21a compared to its wild-type parent [54], that were also introduced by the nonspecific chemical mutagenesis approach. Several of these mutations contribute significantly to the safety of this live oral vaccine, like the mutation identified in *rpoS* [55]. *rpoS* encodes an RNA polymerase sigma factor (sigma s), which is a central regulator of the general stress response.

The live oral vaccine Ty21a (Vivotif®) is manufactured by Berna Biotech (now Johnson & Johnson). The vaccine is produced as an enteric-coated capsule containing 2–6 x 10^9 colony forming units of Ty21a and 5–50 x 10^9 nonviable Ty21a in a lyophilized form. The capsules should be ingested on an empty stomach, following a three-dose or four-dose (recommended in Canada and the United States) immunization schedule with an interval of 2 days between doses.

The Ty21a vaccine should be stored at 4 °C and is therefore dependent on the perpetuation of the cold chain. Storage at elevated temperatures results in a lower number of viable cells and therefore in a decreased potency of the vaccine.

As previously mentioned, the Ty21a attenuated *S.* Typhi strain does not express the Vi antigen. Therefore no Vi specific immune response is raised. However, the vaccine elicits a serum IgG O antigen specific antibody response and it has been shown that seroconversion of O antibodies correlates with protective efficacy [56]. Furthermore vaccination with Ty21a results in mucosal IgA and systemic cell-mediated immune responses against H and O antigens.

Several field trials in Santiago, Chile were conducted which involved around 532'000 children between 6 and 19 years of age. The following findings resulted from these trials: (i) three doses of the enteric-coated vaccine formulation (every other day interval between doses) provided 67 % efficacy for 3 years and over 7 years of follow-up Ty21a conferred 62 % protection [57]; (ii) one or two doses of the enteric-coated Ty21a vaccine resulted in moderate, short lived protection [58]; (iii) four doses of the enteric-coated capsules resulted in a significant lower incidence of typhoid fever compared to the three-dose immunization schedule [59]. Based on this finding the four-dose immunization schedule is recommended in the United States and Canada. Furthermore, trials with the enteric-coated vaccine showed that young children are not as well protected as older children. It is recommended that the vaccine is administered to children older than 6 years of age and that revaccination should occur every 5 years.

The Ty21a vaccine is well tolerated and adverse events are uncommon. Post-marketing surveillance from VAERS reported an overall rate of 9.7 adverse events per 100'000 doses of distributed Ty21a.

New generation vaccines In most endemic areas, the peak incidence of typhoid fever is typically observed in schoolchildren and young adults between 5 and 19 years of age. This would make

it possible to design school-based immunization programs in the developing world using the currently licensed vaccines to control typhoid fever. However, health authorities declined the initiation of such school-based vaccination programs because it would deduct scarce resources from the Expanded Program on Immunization (EPI). The EPI is a World Health Organization (WHO) program with the objective to vaccinate children throughout the world and which in developing world focuses mainly on infants less than 12 months of age [27]. Unfortunately, Ty21a and the purified Vi vaccines are only moderately immunogenic in infants and young children and the immunity elicited would not protect for the years later when the children reach the high-risk years of their lives. New typhoid vaccines that are well tolerated, highly immunogenic in infants, and confer a long term protection would be very beneficial to include in the EPI. New typhoid vaccine candidates are described in the following sections.

New attenuated strains of *S.* Typhi as live oral vaccines

The licensed live oral vaccine Ty21a is only modestly immunogenic and requires three to four initial doses and boosters every 5 years. Furthermore the mutations that attenuate Ty21a are not fully defined [47]. Several attempts have been undertaken to engineer new candidate vaccine strains that are as well tolerated as Ty21a but much more immunogenic, so that a single dose will elicit long-term protective immunity.

The availability of the sequence of the complete genomes of wild-type *S.* Typhi strain Ty2 and CT18 has greatly facilitated the construction of defined genomic mutations using recombinant DNA technology. It became evident, that the choice of the parent wild-type strain greatly influences the characteristics of the derived attenuated vaccine strain. The same mutation in distinct strain backgrounds can have different clinical outcome. To study the attenuating potential of various genomic mutations, *S.* Typhimurium strains have been constructed harbouring these mutations and their virulence was tested in the mice model. However, the phenotype of attenuated *S.* Typhimurium strains in mice does

not exactly reflect the behaviour of the homologous *S.* Typhi strain in humans. The common strategy to engineer a candidate attenuated vaccine is to inactivate genes encoding biochemical pathways, regulatory systems, stress proteins and virulence factors. Based on the results obtained in clinical trials, the most promising vaccine candidates are shortly described below.

CVD908-*htrA* is a derivative from the parent strain CVD908 which harbors precise deletion mutations of *aroC* and *aroD* in the Ty2 background [60,61]. *AroC* and *aroD* are genes encoding enzymes in the aromatic amino acid biosynthesis pathway. Therefore, the auxotrophic *S.* Typhi mutant is dependent on certain metabolic intermediates that are only present in a very low concentration within the host cell. As the name implies, strain CVD908-*htrA* additionally harbors a deletion mutation of *htrA*, which is a stress protein required by many bacterial pathogens to successfully cause infection. HtrA deals with misfolded proteins in the periplasm and it has been shown that an *htrA* mutant of *S.* Typhimurium is greatly attenuated in mice [62]. In clinical trials CVD908-*htrA* was immunogenic and well tolerated, although some adverse effects like mild diarrhea were observed [63-65].

CVD909 is a derivative of CVD908-*htrA* that constitutively expresses the Vi antigen. The licensed live attenuated strain Ty21a lacks the Vi capsular polysaccharide and other live attenuated vaccine candidates, although they express the Vi *in vitro*, did not elicit a Vi specific immune response. However, the purified Vi polysaccharide and the Vi-rEPA conjugate vaccine (described on page 34*ff.*) stimulate serum Vi antibodies that confer protection against typhoid fever. Therefore it was hypothesized that a live oral vaccine that is able to stimulate serum Vi antibodies could confer higher levels of protection. Expression of the Vi polysaccharide is highly regulated and it is linked to environmental signatures such as osmolarity [66]. CVD909 was constructed by replacing the promoter of the Vi biosynthetic operon, *viaB*, by a strong constitutive promoter. This resulted in constitutive expression of the Vi

antigen [67]. A Phase I clinical study showed that CVD909 is well tolerated, but it did not consistently stimulate serum IgG anti-Vi antibodies However, higher dosages elicited mucosal IgA anti-Vi responses [68].

Ty800 is a *phoP* and *phoQ* deletion mutant of Ty2 [69]. In a Phase II clinical trial the vaccine candidate was well tolerated and stimulated serum and mucosal anti-LPS antibody responses.

M01ZH09 harbors deletion mutations in *aroC* and *ssaV* that were introduced into the wild-type strain Ty2 [70]. SsaV is a component of the type III secretion system encoded on the *Salmonella* pathogenicity island 2 (SPI 2), which plays a role in systemic infection and survival in macrophages. Mutations in *ssaV* result in the inability to translocate SPI 2 effector proteins. This vaccine candidate was tested in several dose-ranging trials to evaluate its immunogenicity and to optimize its formulation. The vaccine was well tolerated with only minor adverse effects. 14 of 15 volunteers who ingested a single dose of 10^9 CFU exhibited rises in antibody secreting cells making IgA anti-LPS antibodies. A ≥ 4-fold increase in serum IgG anti-LPS antibodies was observed in 11 of 15 recipients [71]. A phase II study was conducted in Vietnamese children 5 to 14 years of age and M01ZH09 proved to be safe and immunogenic in this study group.

Vi conjugate vaccine: Vi-rEPA Purified polysaccharide vaccines are T-cell independent antigens that do not induce a booster response upon revaccination, and are poor immunogens for young children and infants. Furthermore, T-independent antigens do not establish immunological memory and reinjections are recommended every 3–5 years. These disadvantages can be overcome by conjugating the polysaccharide to a protein carrier. When the polysaccharide is conjugated to a protein it behaves like a T-cell dependent antigen. This strategy has been successfully applied in the development of several licensed conjugate vaccine against *Haemophilus influenza* type B (Hib), pneumococcal, and meningococcal infections [72-76].

As with these other conjugate vaccines the deficiencies of the purified Vi polysaccharide vaccine were overcome by conjugating the capsular sugar of *S.* Typhi to carrier proteins. Szu and colleagues at the National Institute of Health developed a Vi conjugate vaccine candidate for clinical trials consisting of the purified Vi polysaccharide covalently linked to recombinant toxoid variant of exoprotein A (rEPA) of *Pseudomonas aeruginosa*, designated as Vi-rEPA [77]. Vi-rEPA was prepared by treating the carrier protein with adipic acid dihydrazide (ADH) and binding of Vi in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). In animal models it was established that conjugates prepared with the native Vi polysaccharide elicited higher Vi antibody titers than conjugates containing lower molecular weight Vi [78] and that revaccination clearly boost the serum Vi antibody titer.

A dose of the Vi-rEPA conjugate contained 25 μg of Vi polysaccharide and around the same amount of protein in 0.5 ml of isotonic buffer. This vaccine candidate was shown to be safe and immunogenic in U.S. adults before clinical trials were undertaken in highly endemic areas in the Mekong Delta, Vietnam. Phase I and phase II clinical trials demonstrated the safety and immunogenicity of Vi-rEPA in adults (18–35 years), school-age children (5–14 years) and pre-school children (2–4 years) [79]. In the group of adults the serum anti-Vi IgG response was followed for as long as 10 years after the injection. Although the antibody level declined slightly, serum Vi antibody remained more than sevenfold higher than the prevaccination baseline 10 years after vaccination. The group of pre-school children were injected once or twice (6 weeks apart) with the conjugate. Children that were injected with the conjugate vaccine had a significant higher serum anti-Vi IgG titer as the control group that was injected with the purified polysaccharide vaccine and a booster response was observed after the second dose. Furthermore no adverse reactions were detected.

In a double-blind randomized phase III trial, safety, immunogenicity and efficacy of the Vi-rEPA conjugate vaccine were evaluated in children two to five years of age in Dong Thap province, Vietnam [80]. 11'091 children received two injections, six week

apart of either Vi-rEPA or a saline placebo. Cases of typhoid fever, diagnosed by the isolation of *S.* Typhi from blood cultures, were identified by active surveillance over a period of 27 months, and a passive surveillance during an additional 19 months. Less than 2% of children had adverse reactions, and none of these were considered serious. After 27 months of follow-up, *S.* Typhi was isolated from 4 of the 5525 children who were vaccinated with the Vi-rEPA vaccine and from 47 of the 5566 children who received the placebo, resulting in an efficacy of 91.5%. There was no correlation observed between level of efficacy and the children's age nor was a decrease of efficacy observed over time. During the additional 19 months of passive surveillance, typhoid fever was diagnosed in three children of the vaccine group versus 17 cases in the control group [81]. Over the full period of 46 months the overall vaccine efficacy was 89.0%. Additionally, 771 children received only one injection. The efficacy of the vaccine in this group was 87.7% after 46 months of follow-up. A long-term follow-up study to determine the long-term protection and antibody persistence is underway, which should clarify the necessity of a booster dose.

As with other conjugate vaccines a dosage-related immunogenicity was observed in a dosage study of the Vi-rEPA conjugate in children two to five years old in Pho Tho province, Vietnam [82]. Children received two injections, six weeks apart, containing 25 μg, 12.5 μg, or 5 μg of Vi polysaccharide. After one year the serum anti-Vi IgG levels remained significantly higher than the preimmune levels and a correlation between the dosage and immune response was observed. Based on this study a dosage of 25 μg of polysaccharide is recommended.

The Vi-rEPA vaccine was developed for administration to infants as part of their routine immunization. Hence, the safety and immunogenicity of the Vi-rEPA conjugate vaccine was evaluated in a study comprising a total of 301 Vietnamese infants who received Expanded Program of Immunization (EPI) vaccines alone or with Vi-rEPA or *Haemophilus influenzae* type b-tetanus toxoid conjugacte (Hib-TT) at 2, 4, and 6 months of age. A fourth dose of Vi-rEPA or Hib-TT was administered at 12 months. Infants

were visited regularly after each injection to monitor adverse effects and sera were assayed for anti-Vi IgG and antibodies specific for the other vaccine components at 7, 12, and 13 months. This study showed that the Vi-rEPA conjugate is safe in infants, induced protective anti-Vi levels, and was compatible with EPI vaccines [83].

Paratyphoid fever

Typhoid and paratyphoid fever are clinically similar acute febrile illnesses that are referred to as enteric fevers. In the case of paratyphoid fever the causative agent is *Salmonella enterica* subspecies I serovar Paratyphi A, B and C (*S.* Paratyphi A, B and C). However, infections with *S.* Paratyphi C are rare. In the year 2000 it was estimated that paratyphoid fever caused 5.4 million illnesses worldwide [23]. The transmission and the epidemiology of this human restricted pathogen resemble that of *S.* Typhi. Furthermore, antimicrobial resistance is also a major public health problem in *S.* Paratyphi.

The complete genome sequence has been determined for *S.* Paratyphi A strains ATCC9150 and AKU_12601 [84,85]. Sequence analysis of *S.* Typhi and *S.* Paratyphi A suggests that they are much more closely related to each other than to other *S. enterica* serovars. The genomes of *S.* Paratyphi A and *S.* Typhi have independently accumulated many pseudogenes, which is a common feature found in many host restricted pathogenic bacteria.

Since only *S.* Paratyphi C expresses the Vi antigen, Vi-based vaccines are unlikely to protect against paratyphoid fever caused by *S.* Paratyphi A and B. There is some evidence that the typhoid live oral vaccine Ty21a might confer weak cross-protection against *S.* Paratyphi A and B [86,87]. However, there are currently no licensed vaccines against *S.* Paratyphi. The lack of effective vaccines is of great concern, because of the growing importance of *S.* Paratyphi A as a cause of enteric fever. In a number of Asian countries *S.* Paratyphi A appears to be responsible for a growing proportion

of enteric fever whereas *S.* Typhi incidences decline. In Nepal, the proportion of S. Paratyphi A was 32.9% and 62.5% in 2007 and 2008, respectively [88]. There has also been an increase in *S.* Paratyphi A infections in the province of Guangxi, southeastern China after implementing Vi vaccination in 1999 [89]. This trend raises important concerns whether the introduction of a vaccine against *S.* Typhi, in the absence of a vaccine against *S.* Paratyphi, would have an impact at all on enteric fever [90,91].

Konadu and colleagues at the National Institute of Health developed a conjugate vaccine against *S.* Paratyphi A that is currently evaluated in clinical trials [92]. This vaccine candidate consists of the O-specific polysaccharide (O-SP or O antigen) (described on page 45*ff.*) of *S.* Paratyphi A covalently linked to tetanus toxoid (TT) as carrier protein, designated as SPA-TT. The O-SP was purified from LPS, detoxified by acetic acid to remove the lipid A, and activated with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP). The activated O-SP was bound to tetanus toxoid with adipic acid dihydrazide (ADH) as a linker or directly. The safety and immunogenicity of those two conjugates was evaluated in Vietnamese adults, teenagers, and 2–4 year old children in phase I, and phase II clinical trials [93]. None of the volunteers experienced significant side effects. At 4 weeks after injection both conjugates elicited a greater than 4-fold rise in the serum IgG anti-LPS levels in ≥80% of the adult and teenage volunteers. The children received one or two injections, administered 6 weeks apart. A significant rise in serum IgG anti-LPS titer was also observed in this study group after the first injection. However, a second injection did not elicit a booster response. The antibodies raised by SPA-TT were bactericidal against *S.* Paratyphi A *in vitro*.

Nontyphoidal *Salmonella* disease

Nontyphoidal *Salmonella* (NTS) serovars cause a gut-associated self-limiting gastroenteritis in healthy individuals. It is estimated that each year in the United States around 1.5 million persons are in-

fected with NTS, which results in 580 deaths (WHO, 2005). NTS serovars are frequently zoonotic and have broad host range specificity. Therefore, foods of animal origin are the main source for NTS transmission. *S.* Enteritidis and *S.* Typhimurium are the most frequent serovars causing *Salmonella* outbreaks worldwide [94].

Antimicrobials are usually not required for treatment of gastroenteritis caused by NTS but may be required in severe cases that occur in more susceptible patients like young children, elderly and immunocompromised patients. However, the resistance of NTS strains against antibiotics has significantly increased in recent years. Multidrug resistant NTS strains are commonly found in many food animals, like poultry, pigs, and sheep. Resistance is the result of inappropriate and uncontrolled usage of antibiotics in modern intensified farming and food production methods.

NTS have emerged as an important cause of invasive bloodstream infection in sub-Saharan Africa, amongst young children with malaria and malnutrition, and amongst adults with HIV [11]. NTS infection in these particular hosts, referred to as invasive nontyphoidal *Salmonella* (iNTS) disease, causes a nonspecific febrile illness and the case fatality is 22–54% [95]. In sub-Saharan Africa, the most common causes of iNTS disease are the *Salmonella enterica* serovars Typhimurium and Enteritidis. The transmission of iNTS may be from human to human rather than zoonotic.

There are currently no licensed vaccines for nontyphoidal *Salmonella*. Homologous to *S.* Paratyphi A it has been demonstrated that antibodies raised against the O-specific polysaccharide (O-SP) of *S.* Typhimurium (described on page 45*ff.*) are protective in mice and rabbits [96]. Conjugate vaccines consisting of the O-SP of *S.* Typhimurium covalently linked to tetanus toxoid as protein carrier elicited antibodies in mice that were bactericidal and protected the immunized animals against intraperitoneal challenge with *S.* Typhimurium [97]. However, a study of humoral defence against *S.* Typhimurium in HIV-infected Malawian adults demonstrated that high-titer antibodies directed against the LPS are associated with impaired serum killing of *Salmonella* [98]. Accordingly, anti-LPS antibodies are not protective but rather inhibit the

binding of effective antibodies directed against *Salmonella* outer-membrane proteins. These findings have important implications on the strategy of vaccine development against NTS, away from O antigen glycoconjugates, which could potentially cause harm, and towards cell surface protein targets. Identification of antigenic protein targets has recently been done using a *Salmonella* proteomic array, containing over 2'700 proteins, probed with immune sera from *Salmonella*-infected mice and humans. Vaccination with SseB, the most prominent antigenic target identified with this method, significantly protected mice against *Salmonella* infection [99].

Scope of the thesis: A novel approach for conjugate vaccine production

Conjugate vaccines are among the most effective and safe vaccines against bacterial diseases and have been used in humans for over 30 years. As described in the previous sections, several clinical trials have shown that glycoconjugates are promising vaccine candidates to prevent *Salmonella* infections. However, conjugate vaccines are currently produced by a complex manufacturing process, illustrated in **Figure 3A**. Bacteria producing the polysaccharide antigen and the carrier protein are grown separately, the polysaccharide and carrier protein are then each purified and the polysaccharide is chemically linked to the carrier protein, before being further purified. Chemical conjugation has a number of drawbacks: (i) Several purification steps are required, which might result in substantial loss of material throughout the process and thus lower yields; (ii) Chemical coupling is random and the glycoconjugate produced is not a uniform, customized structure, but a mixture of different glycoconjugates, each with potentially different efficacy profile. Furthermore, chemical conjugation is never complete and free polysaccharide may interfere with immune responses to the glycoconjugates. Any small change in the mixture affects the

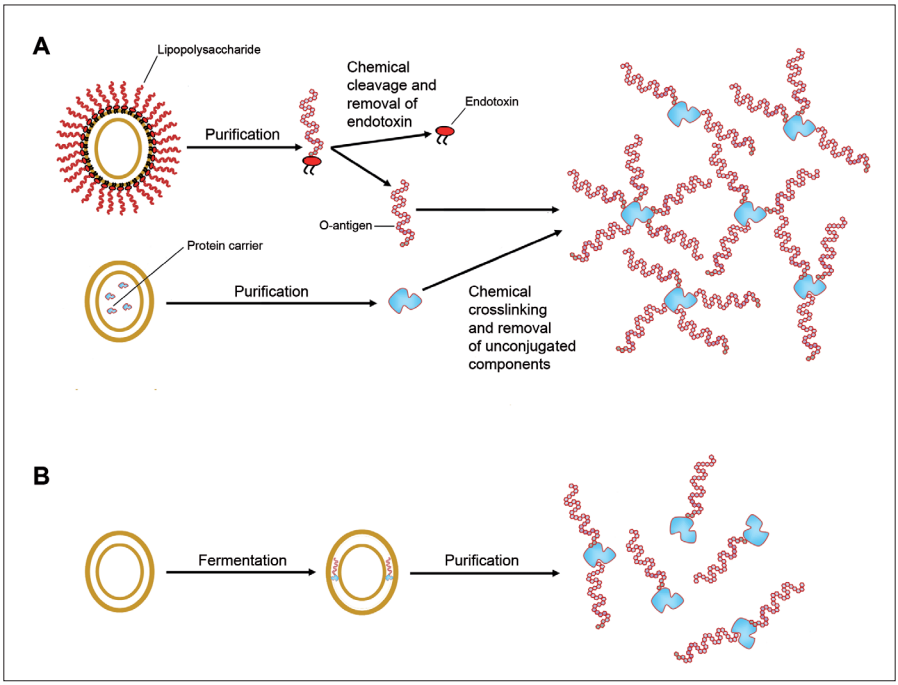


Figure 3: Production of conjugate vaccines.

A Currently, conjugate vaccines are being produced by chemical conjugation of an antigenic polysaccharide and a protein carrier *in vitro*. Therefore, a bacterial strain expressing the carrier protein and the pathogen expressing the polysaccharide structure have to be grown in parallel. Both the protein carrier and the polysaccharide have to be purified separately. Toxic polysaccharides, like LPS, must additionally be detoxified prior to chemical conjugation. The final product has to be further purified from the reaction mixture to remove unconjugated components, which may interfere with immune responses to the glycoconjugates.

B A novel *in vivo* production process for glycoconjugates is based on the bacterial N-linked protein glycosylation system. A production strain is genetically modified so it expresses the antigenic polysaccharide structure, an engineered acceptor protein containing the consensus sequence for N-linked protein glycosylation, and the bacterial oligosaccharyltransferase PglB. After fermentation the final product is extracted form the cell's periplasm and purified.

characteristics of the vaccine, therefore the same mixture must be maintained throughout scale-up and production; (iii) chemical conjugation may affect the structure of both the polysaccharide and the carrier protein, which makes them less immunogenic or, in some cases, not immunogenic at all. Toxic polysaccharides, like LPS, must additionally be chemically detoxified, often leading to further loss of immunogenicity.

A novel approach to produce conjugate vaccines is based on the bacterial N-linked protein glycosylation system. This bacterial protein modification system was first described in the foodborne pathogen *Campylobacter jejuni* [100,101]. The key enzyme of this system, *C. jejuni* oligosaccharyltransferase (PglB), transfers bacterially-synthesized oligosaccharides from a carrier lipid (undecaprenyl pyrophosphate; Und-PP) to periplasmic proteins with an asparagine-containing consensus sequence. This protein glycosylation system was functionally transferred into *Escherichia coli* to produce glycoconjugates *in vivo* [101]. It has been shown that PglB exhibits relaxed substrate specificity towards the glycan moiety and various Und-PP linked polysaccharides can be transferred to proteins [102,103]. Furthermore, it is possible to produce recombinant glycoproteins by inserting the consensus sequence for N-glycosylation (D/E-Y-N-X-S/T, where Y and X can be any amino acid except proline) at multiple desired sites in proteins which are otherwise not glycosylated, like rEPA [104,105]. Therefore, the type of glycoconjugate produced can be tailored by the introduction of genes driving synthesis of the antigenic polysaccharide and of the antigenic protein carrier. After fermentation of the production strain, the glycoconjugate is simply extracted from the periplasm and purified **(Figure 3B)**. This cost-efficient *in vivo* production of glycoconjugates represents an alternative to the conventional manufacturing process.

In this work, the possibilities of using the *in vivo* glycosylation technology for the production of conjugate vaccines against certain *Salmonella enterica* serovars causing human illnesses were exploited. For the development of such vaccines it is crucial to identify antigenic cell surface polysaccharide structures and learn more about

their chemical structure and biosynthetic pathways. The following sections provide an overview of the relevant cell surface polysaccharide structures expressed by *Salmonella enterica* serovars causing human illnesses. Most of these structures have already been targeted for the development of conjugate vaccine candidates using the chemical coupling technology. Furthermore, challenges that might be encountered during development of glycoconjugates using the *in vivo* glycosylation technology are being discussed.

Vi antigen

The causative agent of typhoid fever, *S.* Typhi, expresses a capsular polysaccharide (CPS), known as Vi antigen. Vi expression is largely restricted to *S.* Typhi but it is also found in some strains of *S. enterica* serotype Paratyphi C, *S. enterica* serotype Dublin, and in *Citrobacter freundii*.

In the model organism *Escherichia coli*, CPS are divided into two major groups according to their mechanism of biosynthesis: Group 1 CPS are assembled by a Wzy-dependent pathway, group 2 CPS by an ATP-binding cassette (ABC) transporter-dependent process [106]. The building blocks of group 1 CPS are preassembled on Und-PP at the cytoplasmic leaflet of the inner membrane. These Und-PP-linked subunits are exported across the inner membrane by the putative flippase Wzx and subsequently polymerized at the periplasmic face of the inner membrane by the putative polymerase Wzy. Finally, the CPS is translocated from the periplasm to the cell surface. In contrast, group 2 CPS are completely assembled at the cytoplasmic face of the inner membrane, typically by a processive glycosyltransferase adding activated sugar molecules to the nonreducing end of the nascent chain. The assembled polymer is exported across the inner membrane by an ABC transporter and subsequently translocated to the cell surface. The endogenous acceptor molecule for group 2 CPS biosynthesis is equivocal and the details of initiation and termination of the chain growth have not yet been resolved.

The Vi is a linear, acidic homopolymer of α-1,4-linked N-acetylgalactosaminuronic acid (D-GalNAcA), nonstoichiometrically esterified with acetyl groups at the C-3 **(Figure 4)**. The O-acetyl groups make up most of the surface and immunogenicity of Vi is closely related to the degree of O acetylation [35,36]. Key proteins involved in Vi capsule formation are encoded within a cluster of genes, known historically as the *viaB* locus [107], a region located on a 134 kb DNA island, termed *Salmonella* pathogenicity island 7 (SPI-7) within *S.* Typhi [45]. Precise excision of SPI-7 in Vi-negative mutants has been demonstrated, indicating that Vi antigen expression is located on an unstable region in the *S.* Typhi genome [46]. The *S.* Typhi *viaB* constitutes 10 genes involved in regulation of expression (*tviA*), biosynthesis (*tviB* to *tviE*), and cell surface localization of the Vi polysaccharide (*vexA* to *vexE*). Analysis of the bioinformatics signature of *viaB*, including the presence of a putative ABC transporter and the absence of a homologue of *wzy*/*wzx* respectively, biosynthesis of Vi is thought to be similar to *E. coli* group 2 CPS [106].

Since many important aspects of this group of CPS are equivocal, a molecular characterization of the *viaB* locus encoding the biosynthetic machinery for Vi capsule formation was conducted (see page 53*ff.*). To date, there are no indications that the Vi polysaccharide is assembled on the lipid carrier Und-PP and no periplasmic intermediates have been reported to exist. These are key criteria for being a substrate of the bacterial oligosaccharyltransferase PglB, the key enzyme of the *in vivo* glycoconjugate production technology. However, Und-PP-linked polysaccharides exist in bacteria that resemble the Vi antigen. In the second part of the Results section, polysaccharides that mimic the Vi have been engineered, conjugated, and their immunogenicity has been evaluated in mice for their potential use as a typhoid vaccine component (see page 87*ff.*).

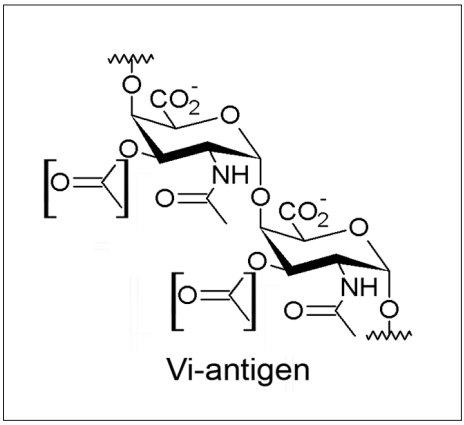


Figure 4: Structure of the Vi antigen.
The Vi is a linear, acidic homopolymer of α-1,4-linked N-acetylgalactosaminuronic acid (D-GalNAcA), variably O-acetylated at C-3.

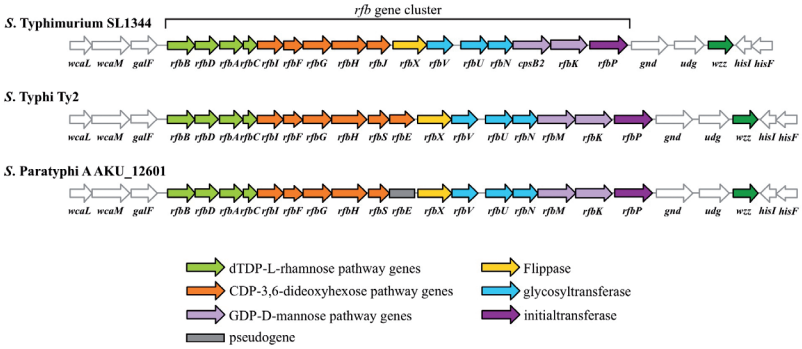
O antigen

As described on page 15*f.* *Salmonella* spp. and other Gram negative bacteria express an O antigen (O-specific polysaccharide, O-SP), which is part of the lipopolysaccharide (LPS). According to the Kauffmann-White scheme, which is based on specific antisera used for cell surface antigen determination, O antigens of the genus *Salmonella* can be classified into groups. For example, *S.* Paratyphi A belongs to the O antigen group A, *S.* Paratyphi B and the nontyphoidal *Salmonella enterica* serovar Typhimurium belong to the O antigen group B, *S.* Enteritidis and *S.* Typhi are grouped into O antigen group D. The O antigen structures of serogroups A, B, and D *Salmonella* share a common trisaccharide backbone illustrated in **Figure 5A**. This structure is recognized by O12 specific antisera, and is therefore associated with antigen factor 12 specificity of the Kauffmann-White scheme. The galactose of the backbone structure can be modified with α-1,6-linked glu-

A

α-R
↓(1→3)
(α-D-Glc)
(1→6)↓(1→4)
[→2)-α-D-Man-(1→4)-α-L-Rha-(1→3)-α-D-Gal-(1→]n

B



C

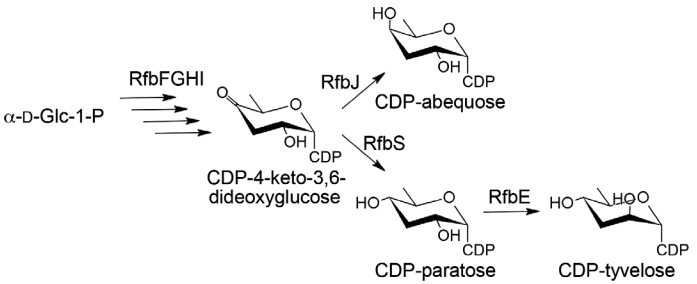


Figure 5: Structure and biosynthesis of the *Salmonella* serogroup A, B and D O antigens.

A The O antigen repeating unit of serogroup A, B and D *Salmonella* are structurally related and share a common backbone trisaccharide structure that might be modified with branching glucoses and O-acetyls. The O antigen serogroups differ in the presence of an immunodominant side-branch R, linked α(1→3) to the mannose of the backbone. Residue R is a dideoxyhe-xose: paratose (3,6-dideoxyglucose) in group A (*e.g. S. Paratyphi A*), abequose (3,6-dideoxygalactose) in group B (*e.g. S. Typhimurium*), and tyvelose (3,6-dideoxymannose) in group D (*e.g. S. Typhi*).

B Enzymes involved in the assembly of the O antigen are encoded within a cluster of genes known historically as the *rfb* locus. Not only are the structures of the O-specific polysaccharides of group A, B and D *Salmonella* very similar, but also their respective *rfb* loci. The *rfb* gene cluster is typically localized between the conserved genes *galF* and *gnd* in the chromosome. The functions of the individual enzymes are indicated by different coloration. Note that the individual *rfb* loci only differ in the organization of genes involved in CDP-3,6-dideoxyhexose biosynthesis. Group B *rfb* loci encode *rfbJ*, whereas this gene is replaced by *rfbS* and *rfbE* in group A and D strains. In group A strains *rfbE* is non functional due to a frameshift mutation.

C RfbJ, RfbS and RfbE are involved in final steps of CDP-3,6-dideoxyhexose biosynthesis, giving rise to CDP-abequose, CDP-paratose and CDP-tyvelose respectively.

cose, which is associated with factor 1. Glucosylation of the backbone galactose at C-4 generates the 12-2 variant [108].

The serogroups differ in the presence of a side-branch R, linked α(1→3) to the mannose of the backbone. Residue R is a 3,6-dideoxyhexose: paratose (3,6-dideoxyglucose) in group A (factor 2 in the Kauffmann-White scheme), abequose (3,6-dideoxygalactose) in group B (factor 4), and tyvelose (3,6-dideoxymannose) in group D (factor 9).

Furthermore, O-acetyl groups are present in some O antigen structures: The rhamnose of the backbone is partially O-acetylated at C-3 for *S.* Paratyphi A (there is no antigen factor assignment for this modification in the Kauffmann-White scheme), and the

abequose residue of *S.* Typhimurium can be O-acetylated at C-2 (factor 5). The O-acetyl groups play an essential role in immunogenicity of the O antigen. For *S.* Paratyphi A, only conjugates with O-acetyls elicited serum IgG anti-LPS with bactericidal activity [93]. Similarly, in *S.* Typhimurium both O-acetyl positive and negative strains can cause illness. However, O-acetylation has a dramatic effect on the structure of the molecule and its immunogenicity [109]. These immunodominant O-acetyl groups are susceptible to chemical treatment and could be lost during chemical conjugation, resulting in an ineffective vaccine.

The enzymes involved in O antigen assembly are encoded within a cluster of genes, historically known as the *rfb* locus **(Figure 5B)**. O polysaccharides are generally assembled on the lipid carrier undecaprenyl pyrophosphate (Und-PP). The O polysaccharides of serogoups A, B, and D *Salmonella* are assembled according to the Wzy-dependent biosynthesis pathway (reviewed in [110]). The first step in this pathway involves the reversible transfer of galactose-1-P from UDP-galactose to undecaprenyl phosphate (Und-P) resulting in the formation of Und-PP-linked galactose. This reaction is catalyzed by the enzyme WbaP (formerly RfbP). Subsequently, *rfb* locus encoded glycosyltransferases assemble the O repeating unit at the inner face of the cytoplasmic membrane. Following their assembly, the Und-PP-linked O units are transported across the cytoplasmic membrane to the periplasmic side by the flippase Wzx (formerly RfbX). At the periplasmic face of the cytoplasmic membrane Und-PP-linked O units are polymerized by the O polysaccharide polymerase Wzy. Surprisingly, Wzy is not encoded in the *rfb* locus of *Salmonella*. The reaction of polymerization involves the transfer of nascent polymer from its Und-PP carrier to the nonreducing end of a new Und-PP-linked O repeating unit. The chain length of Wzy-dependent O antigens is regulated. The Wzz protein generates a modal distribution of O polysaccharide chain lengths. Several *S. enterica* serovars have two *wzz* genes, resulting in a bimodal distribution of chain lengths. Finally, the assembled O polysaccharide is transferred from Und-PP and ligated to the lipid A-core by the O antigen ligase WaaL.

Enzymes responsible for O-acetylation of the *Salmonella* O polysaccharide backbone structure, illustrated in Figure 5A, are encoded outside the *rfb* locus [111]. Similarly, enzymes involved in glucosylation are not encoded within the *rfb* locus. The branching glucose residues are typically added in the periplasm after polymerization of the O repeat units by a prophage encoded system, which is found in many other species like *Shigella flexneri* (reviewed in [112]). Three genes are typically involved in glucosylation of the O antigen repeat: *gtrA, B,* and *gtr(type)*. The products of the first two genes are highly conserved between the different glucosylation systems, whereas the third appears to be unique. GtrB contains motifs that are also found in Alg5 of *Saccharomyces cerevisiae*, a well characterized dolichyl phosphate glucosyltransferase. It is proposed that GtrB catalyzes the transfer of glucose from UDP-glucose to undecaprenyl phosphate (Und-P) at the cytosolic side of the inner membrane, resulting in the formation of Und-P-glucose [113,114]. This lipid linked glucose is then flipped across the cytosolic membrane by the putative flippase GtrA, before the glucosyl residue is transferred to the growing O antigen chain by the glucosyltransferase Gtr(type). This system resembles the biosynthesis of the eukaryotic N-glycan oligosaccharide in the lumen of the endoplasmatic reticulum, where mannosyl and glucosyl residues are transferred from dolichyl phosphate (Dol-P)-mannose and Dol-P-glucose precursors. The prophage encoded system involved in glucosylation of the *S*. Typhimurium O antigen galactose (C-4 position) has been identified [108]. The glucosyltransferase involved in C-6 modification of the O antigen galactose has not yet been described. However, BLAST search identifies two GtrB homologous in the *S*. Typhimurium LT2 genome: STM0558 [108] and STM4205. The latter genomic region might be responsible for C-6 glucosylation.

Not only are the O antigen structures of serogroups A, B, and D *Salmonella* very similar, but also their respective *rfb* gene clusters, which differ only in genes for the biosynthetic pathways of the immunodominant 3,6-dideoxyhexoses. The gene *rfbJ* found in group B strains encodes abequose synthase, which catalyzes the conver-

sion of CDP-4-keto-3,6-dideoxy-D-glucose to CDP-abequose. In group A and D strains *rfbJ* is replaced by *rfbS* and *rfbE*. RfbS catalyzes the conversion of CDP-4-keto-3,6-dideoxy-D-glucose to CDP-paratose and RfbE is a C-2 epimerase, catalyzing the final step of CDP-tyvelose formation **(Figure 5C)** [115]. However, *S.* Paratyphi A contains a frameshift mutation in *rfbE*, resulting in pseudogene formation and the inability to convert CDP-paratose to CDP-tyvelose.

As described in previous sections conjugate vaccine candidates have been developed against *S.* Paratyphi A and *S.* Typhimurium, both consisting of the O-specific polysaccharide covalently linked to a protein carrier. The presence of chemical instable O-acetyl groups in these O-SPs would favor the *in vivo* glycoconjugate production technology. However, a commonality of the vaccine relevant *Salmonella* O-SPs is a reducing end galactose. The PglB-mediated transfer of such polysaccharide structures has not yet been observed, and it was proposed that only glycans containing an acetamido group at the C-2 position of the reducing end saccharide serve as substrates to the bacterial OST [103]. In the third part of the results section, the substrate specificity of the bacterial oligosaccharyltransferase PglB was explored and the transfer of a *Salmonella* O-SP to an acceptor protein was investigated (see page 117*ff.*). In this work, the *S.* Typhimurium O-SP was used as a model. However, if it would be possible to produce such a glycoconjugate it would quickly be adaptable to other important *Salmonella enterica* serovars causing human illnesses due to the close relatedness of their O-SP structures.

Molecular characterization of the *viaB* locus encoding the biosynthetic machinery for Vi capsule formation in *Salmonella* Typhi

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Contributions

Cloning and expression of *viaB* locus/mutant derivatives
Vi localization/quantification by blot, agglutination, ELISA
GroEL detection
Radiolabeling of Vi
Size exclusion chromatography of Vi
O-acetyl content determination

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Abstract

The Vi capsular polysaccharide (CPS) of *Salmonella enterica* serovar Typhi, the cause of human typhoid, is important for infectivity and virulence. The Vi biosynthetic machinery is encoded within the *viaB* locus composed of 10 genes involved in regulation of expression (*tviA*), polymer synthesis (*tviB-tviE*), and cell surface localization of the CPS (*vexA-vexE*). We cloned the *viaB* locus from *S.* Typhi and transposon insertion mutants of individual *viaB* genes were characterized in *Escherichia coli* DH5α. Phenotype analysis of *viaB* mutants revealed that *tviB*, *tviC*, *tviD* and *tviE* are involved in Vi polymer synthesis. Furthermore, expression of *tviB-tviE* in *E. coli* DH5α directed the synthesis of cytoplasmic Vi antigen. Mutants of the ABC transporter genes *vexBC* and the polysaccharide copolymerase gene *vexD* accumulated the Vi polymer within the cytoplasm and productivity in these mutants was greatly reduced. In contrast, *de novo* synthesis of Vi polymer in the export deficient *vexA* mutant was comparable to wild-type cells, with drastic effects on cell stability. *VexE* mutant cells exported the Vi, but the CPS was not retained at the cell surface. The secreted polymer of a *vexE* mutant had different physical characteristics compared to the wild-type Vi.

Introduction

The causative agent of the human systemic infection typhoid fever, *Salmonella enterica* subspecies I serotype Typhi (*S.* Typhi), expresses a capsular polysaccharide (CPS) known as Vi antigen (Vi). The Vi capsule provides *S.* Typhi with mechanisms to avoid host defenses [116,117] and it is important in enhancing infectivity and virulence [12,47,49]. Besides being an important virulence factor, the Vi is also a protective antigen and a vaccine based on purified Vi polysaccharide has been developed and licensed for use as a parenteral vaccine against typhoid fever [118].

Vi is a linear, acidic homopolymer of α-1,4-linked *N*-acetylgalactosaminuronate (D-GalNAcA), variably O-acetylated at C-3. The O-acetyl groups make up most of the surface and the immunogenicity of Vi is closely related to the degree of O acetylation [35,36]. Key proteins involved in Vi capsule formation are encoded within a cluster of genes, known historically as the *viaB* locus [107], a region located on a 134 kb DNA island, termed *Salmonella* pathogenicity island 7 (SPI-7) within *S.* Typhi [45]. The *S.* Typhi *viaB* constitutes 10 genes involved in regulation of expression (*tviA*), biosynthesis (*tviB* to *tviE*), and cell surface localization of the Vi polysaccharide (*vexA* to *vexE*) **(Figure 1A)**. Analysis of the bioinformatics signature of *viaB* highlighted the presence of a putative ATP-binding cassette (ABC) transporter and the absence of a homologue of *wzy*/*wzx* respectively. Therefore, biosynthesis of Vi is thought to be similar to *E. coli* group 2 CPS [106].

Vi expression in *S.* Typhi is under control of the RcsB-RcsC and OmpR-EnvZ two-component regulator systems [119-121] and when the complete *viaB* operon is expressed in *Escherichia coli* K12 a similar pattern of regulation can also be observed [122]. RcsB-RcsC and OmpR-EnvZ likely interact with TviA and regions upstream of the *tviA* promoter thereby linking Vi expression to environmental signatures such as osmolarity [66]. TviA is an activator of the *viaB* operon and deletion of the *tviA* gene strongly decreases expression of the Vi capsule [123].

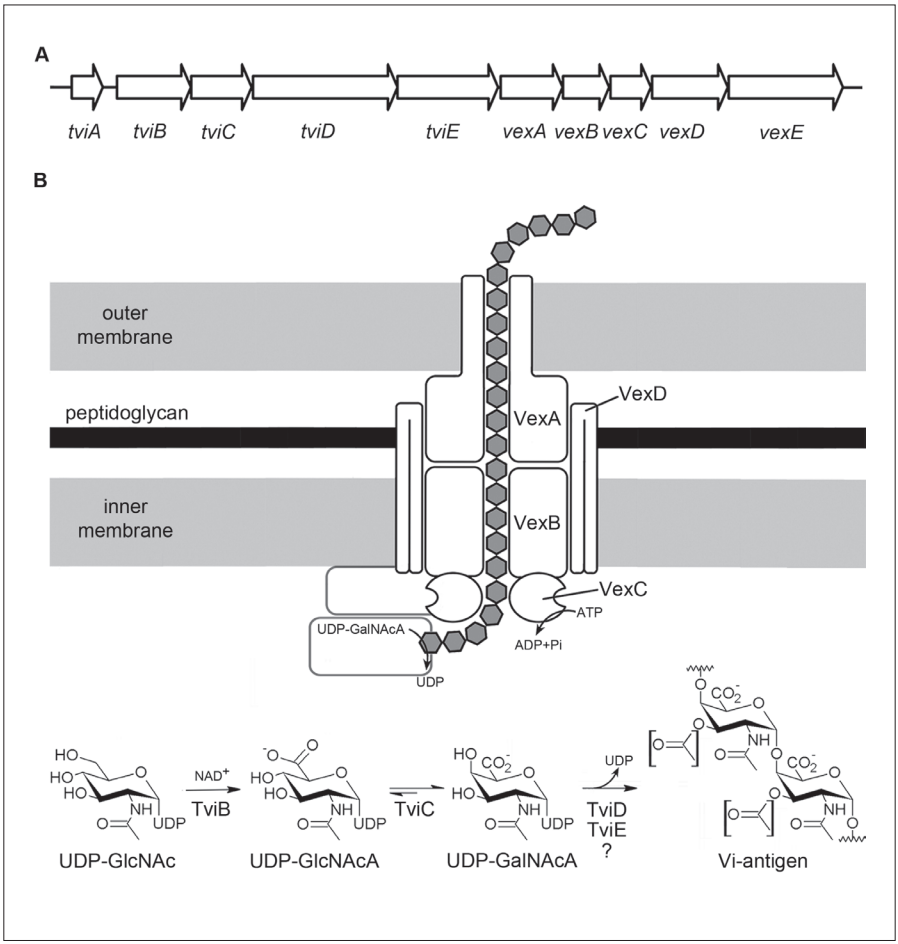

Figure 1: Overview of the *viaB* operon and Vi capsular polysaccharide biosynthetic pathway.

A Schematic diagram of the *S.* Typhi *viaB* operon.

B The Vi antigen is a linear polymer of α-1,4-linked N-acetylgalatosaminuronate, nonstoichiometrically esterified with acetyl groups at the C-3. A TviB-catalyzed oxidation of UDP-N-acetylglucosamine (UDP-GlcNAc) followed by the TviC-catalyzed epimerization at the C-4 of UDP-N-acetylglucosaminuronate (UDP-GlcNAcA) results in the formation of UDP-N-acetyl-

galactosaminuronate (UDP-GalNAcA), the building block for Vi polymer formation. The Vi polymer is synthesized in the cytoplasm and assembly is dependent on TviD and TviE. The enzyme catalyzing the O-acetylation of the capsular polysaccharide has not yet been identified. Subsequent translocation of the polysaccharide to the cell surface follows an ATP-binding cassette (ABC) transporter-dependent process. The transporter consists of VexA, VexB, VexC and VexD. The precise function of VexE is equivocal although it might be involved in anchoring the Vi to the cell surface.

Biosynthesis of the Vi polysaccharide takes place in the cytoplasm and requires functional TviB, TviC, TviD and TviE proteins [123] **(Figure 1B)**. TviB and TviC are involved in catalyzing the conversion of UDP-*N*-acetylglucosamine (UDP-GlcNAc) to UDP-*N*-acetylgalactosaminuronate (UDP-GalNAcA), which serves as the building block for the assembly of the Vi polymer [124]. Assembly of the Vi polymer is dependent on functional expression of *tviD* and *tviE* [123]. The enzyme responsible for O-acetylation of the Vi polysaccharide has not yet been identified.

Cell surface localization of the Vi polymer is dependent on functional expression of *vexA*, *vexB*, *vexC*, *vexD* and *vexE* **(Figure 1B)**. The predicted lipoprotein VexA belongs to group B of the outer membrane polysaccharide export (OPX) proteins [125]. OPX proteins for which a high-resolution structures have been solved include Wza [126], a protein essential for group 1 CPS expression on the surface of *E. coli* and the *E. coli* group 4 capsule protein GfcC [127]. The Wza protein forms an octameric structure that spans the outer membrane and protrudes into the periplasm, thereby forming a water-filled channel. One of the most extensively studied bacterial group 2 capsules is the K1 serotype of *E. coli*. The *E. coli* K1 capsular gene cluster encodes KpsD, which is the functional homologue of VexA. VexBC belong to the large family of ABC transporters whose members have been implicated in the transport of substrates across membranes. The *E. coli* K1 ABC transporter KpsMT is a close homologue of VexBC [128]. KpsM is a hydrophobic, integral inner membrane protein with six

transmembrane domains whereas KpsT is a hydrophilic, peripheral inner membrane protein containing an ATP-binding domain. The functional transporter is proposed to consist of two subunits each of KpsM and KpsT. VexD and its homologous protein KpsE of *E. coli* K1 belong to a family called the polysaccharide copolymerases subfamily 3 (PCP-3) [125]. There is no structural information yet available about this subfamily but all PCP proteins have a characteristic membrane topology in which a large periplasmic loop is flanked by two transmembrane regions localized in the inner membrane. PCP-3 proteins might provide a periplasmic scaffold for linking the ABC transporter in the inner membrane with the OPX protein in the outer membrane therefore assembling the complete polysaccharide translocation machinery. VexE seems to be responsible for anchoring the Vi to the cell surface [123].

To investigate the function of individual proteins involved in biosynthesis and cell surface expression of the Vi CPS in greater detail, the *viaB* cluster was cloned from *S.* Typhi and transposon insertion mutants of individual *viaB* genes were characterized in *E. coli* DH5α. A detailed and comprehensive phenotype characterization of *viaB* single gene mutants and their effect on biosynthesis and cell surface expression of the Vi CPS is reported here.

Results

Expression of the Vi capsule in *E. coli* DH5α

The low copy plasmid pGVXN158 was constructed using a 14.9 kb DNA fragment of *S.* Typhi BRD948 that harbors the 10 open reading frames of the *viaB* operon with around 900 bp upstream of the first gene *tviA*. Therefore the *viaB* operon contains the natural regulatory sequences and expression is not controlled by elements encoded on the plasmid backbone. *E. coli* DH5α was transformed with pGVXN158 whereupon the transformants changed colony morphology towards a smooth colony appearance, indicating the expression of a capsule. These cells could be agglutinated

using a Vi specific antibody. Furthermore, these encapsulated cells were tested for susceptibility to infection by well characterized Vi phages that are part of the classical typing set [129-133]. All 7 Vi phage types were able to infect *E. coli* DH5α(pGVXN158) but not the plasmid free controls. Thus, using these simple methodologies the Vi produced by *E. coli* was indistinguishable from Vi expressed by the parenteral *S.* Typhi. Therefore, this Vi expression system harbored in *E. coli* DH5α was used for further analysis of the biosynthetic machinery assembling the Vi capsule.

Quantification and localization of the Vi in *E. coli* DH5α(pGVXN158) harboring single gene transposon insertions within *viaB* genes

Next the role of the individual genes of the *viaB* operon in Vi capsule expression was analyzed. For this purpose single genes within the *viaB* operon encoded on pGVXN158 were inactivated by Tn*5*-mediated transposon insertional mutagenesis. To this end, *viaB* single gene insertion mutants in all of the genes except the regulator *tviA* were selected and the insertion site was mapped **(Table I)**. Washed cell pellets and the corresponding supernatants of overnight cultures from individual transposon insertion mutants were spotted onto nitrocellulose membranes and the presence of the Vi CPS was detected by immunoblotting using a monoclonal Vi specific antibody. To exclude polar effects on downstream genes, *tviB*::Tn*5*, *tviC*::Tn*5*, *tviD*::Tn*5*, *tviE*::Tn*5*, *vexB*::Tn*5*, *vexC*::Tn*5* transposon insertion mutants were complemented with the corresponding gene cloned and Vi expression was restored (data not shown). Detection of Vi polysaccharide in the individual *E. coli* DH5α(pGVXN158) transposon mutant derivatives revealed three distinguishable phenotypes **(Figure 2A)**: i) a strong signal was detected with cell cultures from *E. coli* DH5α(pGVXN158) and *E. coli* DH5α(pGVXN158) harboring *vexA*::Tn*5* (pGVXN158$_{vexA::Tn5}$) ii) an intermediate

Table I

Overview of transposon insertion mutants analyzed in this study.

Plasmid	Tn5 insertion site*	Immunoblotting	Agglutination	Phage infection**
pGVXN157	–	–	–	–
pGVXN158	–	+++	+	+
pGVXN158*tviB*::Tn*5*	679/ 1278	–	–	nd
pGVXN158*tviC*::Tn*5*	268/ 1047	–	–	nd
pGVXN158*tviD*::Tn*5*	1909/ 2496	–	–	nd
pGVXN158*tviE*::Tn*5*	388/ 1737	–	–	nd
pGVXN158*vexA*::Tn*5*	350/ 1068	++	–	-
pGVXN158*vexB*::Tn*5*	679/ 795	+	–	nd
pGVXN158*vexC*::Tn*5*	515/ 696	+	–	–
pGVXN158*vexD*::Tn*5*	820/ 1305	+	–	nd
pGVXN158*vexE*::Tn*5*	1247/ 1971	–	–	–

* bp of insertion site from 3' end of the gene/ total length of gene

** nd: not determined

staining was associated with *vexB*::Tn*5* (pGVXN158*vexB*::Tn*5*), *vexC*::Tn*5* (pGVXN158*vexC*::Tn*5*), and *vexD*::Tn*5* (pGVXN-158*vexD*::Tn*5*), whereas iii) no Vi was detected in cell cultures of *E. coli* DH5α carrying the empty plasmid control pGVXN157 or *E. coli* DH5α(pGVXN158) harboring *tviB*::Tn*5* (pGVXN158-*tviB*::Tn*5*), *tviC*::Tn*5* (pGVXN158*tviC*::Tn*5*), *tviD*::Tn*5* (pGV-XN158*tviD*::Tn*5*), *tviE*::Tn*5* (pGVXN158*tviE*::Tn*5*) or *vexE*::T-n*5* (pGVXN158*vexE*::Tn*5*). To determine if the detection of Vi observed in the *vexA*::Tn*5*, *vexB*::Tn*5*, *vexC*::Tn*5* or *vexD*::Tn*5* transposon insertion mutants was associated with surface expression of capsular Vi, slide agglutination and Vi phage infections were performed **(Table I)**. None of the cells harboring these transposon insertion mutants could be agglutinated with a Vi specific antibody and none of the tested mutants could be infected with either one of the seven Vi phages. Taken together these results indicate that Vi

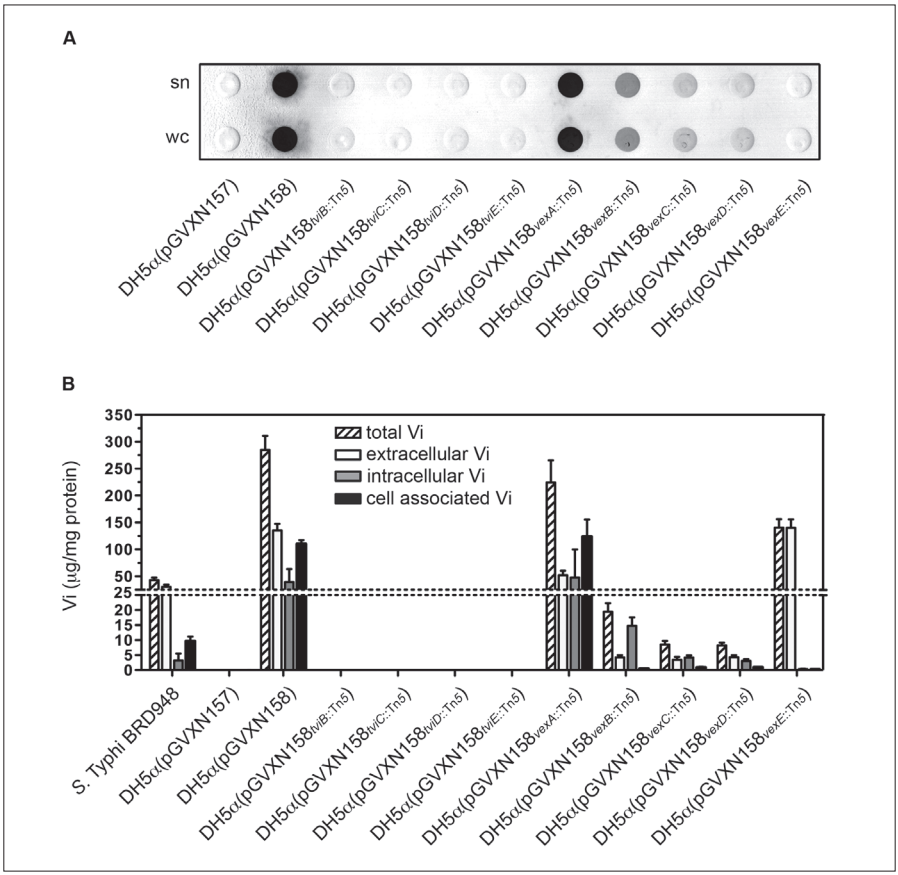

Figure 2: Localization and quantification of the Vi polysaccharide in different *viaB* transposon insertion mutants.

A Washed cell pellets (wc) and the corresponding supernatants (sn) of DH5α overnight cultures harboring the indicated plasmids were spotted onto nitrocellulose and blotted with anti-Vi antiserum.

B Vi content was measured in cell culture supernatants (extracellular Vi), in a suspension of washed and fixed cells (cell associated Vi), and in cell lysates using a quantitative sandwich ELISA. Intracellular Vi was defined using the difference in Vi measured in cell lysates and fixed cells. The amount of Vi was normalized to the protein content measured in the cell pellets.

polysaccharide is still produced in cells expressing the *viaB* locus with a transposon insertion in *vexA*, *vexB*, *vexC* and *vexD* but this is not cell surface associated.

To generate a more detailed view about the quantities and the localization of the Vi polysaccharide produced in the different *viaB* insertion mutants, a sandwich ELISA was developed. With this method Vi polysaccharide contents were measured in the cell culture supernatant (extracellular Vi), in a suspension of washed cells that were fixed with paraformaldehyde (cell associated Vi), and in cell lysates. Intracellular Vi was defined using the difference in Vi measured in cell lysates and fixed cells **(Figure 2B)**.

E. coli DH5α(pGVXN158) cells expressed 6–7 times more Vi than *S.* Typhi BRD948, although the cellular distribution of Vi was similar. Vi was mainly detected on the cell surface, and in the cell culture supernatant. No Vi was detected in DH5α-(pGVXN158*tviB*::Tn*5*), DH5α(pGVXN158*tviC*::Tn*5*), DH5α-(pGVXN158*tviD*::Tn*5*), DH5α(pGVXN158*tviE*::Tn*5*) or DH5α-(pGVXN157). These data confirm the results by Virlogeux *et al.* [123] that TviB, TviC, TviD and TviE are involved in the biosynthesis of the Vi polysaccharide. Vi distribution looked similar in DH5α(pGVXN158*vexB*::Tn*5*), DH5α(pGVXN158*vexC*::Tn*5*) or DH5α(pGVXN158*vexD*::Tn*5*) whereas these insertion mutants produced 15–35 times less Vi in total as compared to DH5α-(pGVXN158). Vi in these mutants was mainly detected predominantly inside the cells and in the culture supernatants. In contrast, DH5α(pGVXN158*vexA*::Tn*5*) and DH5α(pGVXN158*vexE*::Tn*5*) produced large amounts of Vi the former in the same magnitude range as DH5α(pGVXN158) whereas the *vexE*::Tn*5* mutant produced approximately half the amount of the total Vi measured in DH5α cells expressing the wild type *viaB* locus. However, cellular distribution of the Vi in these two insertion mutants was different. In DH5α(pGVXN158*vexA*::Tn*5*) cultures, slightly lower amounts of Vi were detected in the cell culture supernatant, whereas comparable amounts of intracellular and cell associated Vi was measured. The detection of surface exposed Vi in this *viaB* insertion mutant is potentially in conflict with the observed re-

sistance of this strain to Vi phage infection and the fact that it is not agglutinable with a Vi antigen specific antibody. The Vi produced by cells harboring a *vexE* transposon insertion plasmid was mainly localized in the cell culture supernatant with similar amounts comparable to DH5α(pGVXN158). However, in contrast to DH5α(pGVXN158), only low levels of intracellular and cell surface associated Vi was detectable. This result indicates that the *vexE* mutant expresses a functional Vi translocation machinery, but once the polymer is exported, the Vi is not efficiently retained on the cell surface.

Characterization of *vex* transposon insertion mutants by transmission electron microscopy and immunogold labeling of the Vi

In addition to the Vi quantification and localization studies by ELISA, several selected *vex* transposon insertion mutants were analyzed by transmission electron microscopy (TEM) and immunogold labeling of the Vi polysaccharide using the monoclonal anti-Vi antibody P2B1G2/A9 [134]. The specificity of the antibody was tested by immunogold labeling of DH5α cells carrying the empty plasmid pGVXN157 **(Figure 3E)**. These cells do not express the Vi polysaccharide and practically no staining was observed. **Figure 3A** shows the normal cellular distribution of the Vi antigen in DH5α(pGVXN158). The images show encapsulated bacteria where the polysaccharide is mainly localized at the cell surface, but Vi antigen is also detectable in the periplasmic space and inside inclusion-like bodies within the cytoplasm.

As expected, the Vi polysaccharide was differently distributed within the cells carrying *viaB* transposon insertion mutant plasmids. In cells harboring *vexA*::Tn*5* encoding a nonfunctional outer membrane polysaccharide export (OPX) protein, the Vi was mainly stained within large inclusion-like bodies and sometimes clustered at the inside of the inner membrane **(Figure 3B)**. Vi accu-

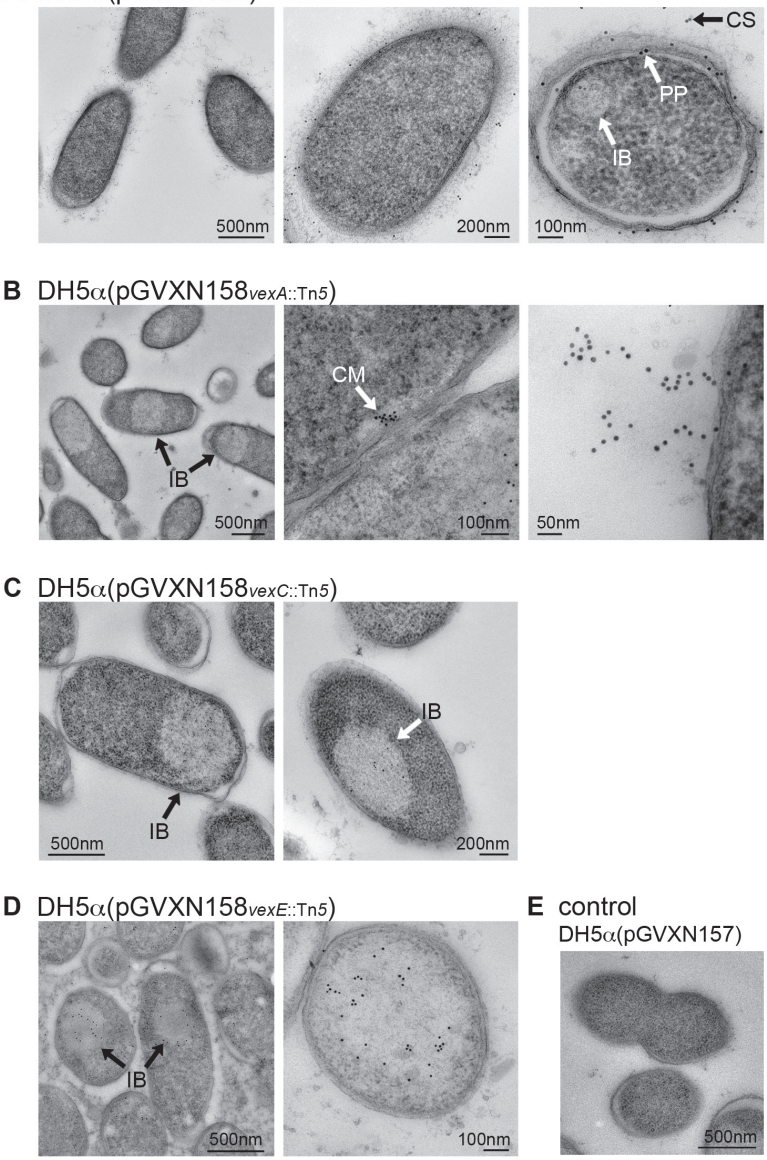
A DH5α(pGVXN158)
500nm
200nm
CS
PP
IB
100nm
B DH5α(pGVXN158vexA::Tn5)
IB
500nm
CM
100nm
50nm
C DH5α(pGVXN158vexC::Tn5)
IB
500nm
IB
200nm
D DH5α(pGVXN158vexE::Tn5)
IB
500nm
100nm
E control
DH5α(pGVXN157)
500nm

Figure 3: Transmission electron micrographs of ultrathin sections from *E. coli* DH5· expressing the Vi polysaccharide.
The Vi has been labeled with the immunogold technique by using an anti-Vi antibody.

A *E. coli* DH5α cells expressing the wild-type *viaB* operon (pGVXN158) are shown. The Vi is mainly localized on the cell surface (CS) but is also stained in the periplasmic space (PP) and inside inclusion-like bodies within the cytoplasm (IB).

B *E. coli* DH5α cells containing the *viaB* operon with a transposon insertion in *vexA* (pGVXN158*vexA*::Tn*5*), the outer membrane polysaccharide export (OPX) protein, are shown. Vi was mainly stained within large inclusion-like bodies (IB) and sometimes clustered at the inside of the inner membrane (CM). Very occasionally, filamentous Vi erupting from the cell surface was labeled.

C *E. coli* DH5α cells containing the *viaB* operon with a transposon insertion in *vexC* (pGVXN158*vexC*::Tn*5*), the ATP-binding protein of the ABC transporter, are shown. The Vi polysaccharide was labeled in large inclusion bodies in the cytoplasm.

D *E. coli* DH5α cells containing the *viaB* operon with a transposon insertion in *vexE* (pGVXN158*vexE*::Tn*5*) are shown. The Vi polysaccharide was labeled in large inclusion bodies in the cytoplasm.

E As a control *E. coli* DH5α cells containing the empty plasmid pGVXN157 are shown.

mulation at the cytoplasmic membrane might indicate the site of Vi translocation over the inner membrane or an accumulation due at a blocked pore complex. Very occasional labeling of filamentous Vi erupting externally from the cell surface was observed.

In cells expressing a nonfuctional ABC transporter ATP-binding protein VexC, DH5α(pGVXN158*vexC*::Tn*5*), the Vi was labeled in large inclusion-like bodies inside the cells **(Figure 3C)**. Disruption of the transport motor in this mutant appears to completely block Vi translocation through the pore complex.

Cells harboring the *vexE*::Tn*5* plasmid display a similar phenotype as seen in the *vexC*::Tn*5* insertion mutant **(Figure 3D)**. Vi was mainly stained within inclusion-like bodies inside the cells. The extracellular Vi detected by ELISA was not visualized in the extracellular spaces.

Defect of the outer membrane polysaccharide export (OPX) protein VexA results in cell instability

It was unexpected to detect large amounts of Vi in the cell culture supernatant and on the cell surface of *E. coli* harboring the *vexA*::Tn*5* mutant derivative. In TEM only very occasionally cell surface associated Vi could be detected **(Figure 3B)** whereas using ELISA and immunoblotting **(Figure 2)** nearly wild-type levels of surface exposed capsular sugar were detected. One hypothesis to explain these observations would be, that cells have a functional transporter in the inner membrane but lack the outer membrane pore might show potential leakiness, therefore releasing the Vi from these instable cells. To test this hypothesis we analyzed the presence of a cytosolic protein, GroEL, in the cell culture supernatant of individual *vex* mutants. Therefore, cells of an overnight culture equivalent to an A_{600} of 1 were collected and proteins were precipitated from the supernatant with trichloroacetic acid. Samples that were hereby normalized to the optical density of the overnight culture were separated by SDS-PAGE and GroEL was detected by western blot using an anti-GroEL antibody **(Figure 4)**. As expected, GroEL was mainly detected in the cell pellet and not in the cell culture supernatant of *E. coli* DH5α(pGVXN158). There were only traces of GroEL detectable in the supernatants of DH5α(pGVXN158*tviB*::Tn*5*), DH5α(pGVXN158*vexB*::Tn*5*), DH5α(pGVXN158*vexC*::Tn*5*), DH5α(pGVXN158*vexD*::Tn*5*) and DH5α(pGVXN158*vexE*::Tn*5*). In contrast, large amounts of GroEL were detected in the supernatant of DH5α(pGVXN-158*vexA*::Tn*5*) cultures. These data indicate that in this mutant derivative a propensity for cell instability does exist. Therefore, the Vi detectable in the ELISA gives an impression of surface associated polysaccharide, when in fact the Vi originated from lysed cells. This explanation is also in agreement with the inability to agglutinate DH5α(pGVXN158*vexA*::Tn*5*) cells, or infect these cells with a Vi phage.

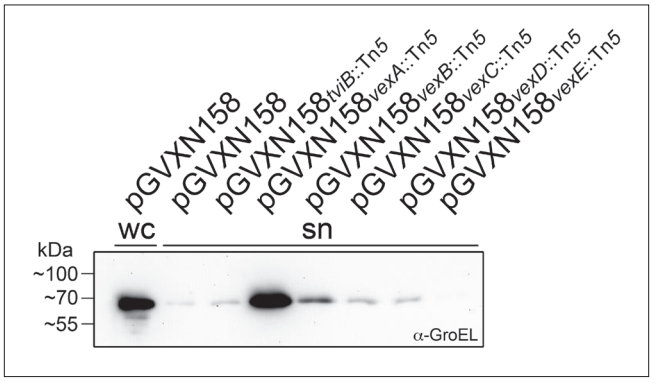

Figure 4: Detection of the cytosolic protein GroEL in cell culture supernatants of individual *vex* mutants.
Either whole cells (wc) or supernatants (sn) of DH5α cell cultures carrying the indicated plasmids were separated by SDS-PAGE and after transfer to a nitrocellulose membrane, GroEL was detected by western blot using an anti-GroEL antibody. Supernatant samples were normalized by TCA precipitation to the optical density of the cell culture. In DH5α cells expressing the wild-type *viaB* operon (pGVXN158), GroEL is mainly detected in the cell pellet. In contrast, the supernatant of a *vexA* transposon insertion mutant (pGVXN-158*vexA*::Tn*5*) contains substantial amounts of the cytosolic marker protein, indicating cell instability.

Size exclusion chromatography of exported Vi produced by *E. coli* DH5α(pGVXN158) and a *vexE*::Tn*5* transposon insertion mutant

The observed inability of Vi expressed by DH5α(pGVXN158-*vexE*::Tn*5*) cells to bind to nitrocellulose membranes or form a capsule once transported to the cell surface lead to the hypothesis, that this polymer might have different physical properties compared to the wild-type polysaccharide. To further characterize these different Vi polymer species we radiolabeled the Vi and separated the purified

polymers by size exclusion chromatography. DH5α(pGVXN157), DH5α(pGVXN158) or DH5α(pGVXN158*vexE*::Tn*5*) cells were metabolically labeled with [^{3}H]GlcNAc and Vi polysaccharide was purified from the cell culture supernatants. There was around 600–700 times more incorporation of radioisotopes into samples purified from cell cultures expressing Vi than from cells carrying the empty plasmid, indicating that the Vi polysaccharide has been labeled specifically **(Table II)**. Subsequently, [^{3}H]Vi was analyzed by size exclusion chromatography using a Sephacryl S-1000 column. The elution profile revealed that the Vi produced by the *vexE*::Tn*5* mutant derivative is clearly distinguishable from the one produced by *E. coli* DH5α(pGVXN158) **(Figure 5)**. The wild-type Vi appears to have a uniform size distribution and the molecular size of the Vi polysaccharide was estimated to be 2 x 10^3 kDa against dextran standards. The Vi polymer secreted by the *vexE*::Tn*5* mutant derivative displayed a tailing of the peak in the earlier elution fractions indicating the presence of either longer polysaccharide chains or the presence of a polymer with a different Stokes radius.

O-acetyl content of the Vi polysaccharide

The ability of a Vi phage to infect a cell is dependent on the expression of a Vi capsule that is partially O-acetylated at the C-3 position of the *N*-acetylgalactosaminuronic acid polymer [135]. The bacterial enzyme responsible for this Vi polysaccharide modification has not yet been identified. PFAM analysis and other bioinformatics interrogations of the proteins encoded in the *viaB* locus did not identify a potential acetyltransferase gene. This suggests that any acetylase is located outside the *viaB* operon. The observation that a Vi phage is able to infect DH5α(pGVXN158) cells implies, that it is well O-acetylated. Furthermore, we speculated that the different elution profiles observed in the size exclusion chromatographic analysis of Vi produced by *E. coli* DH5α(pGVXN158) and a *vexE*::Tn*5*

Table II
Incorporation of [^{3}H] into purified samples (counts/total mg protein).

Strain	cpm mg^{-1} protein
DH5α(pGVXN157)	459.1
DH5α(pGVXN158)	325'984.4
DH5α(pGVXN158*vexE*::Tn*5*)	281'563.9

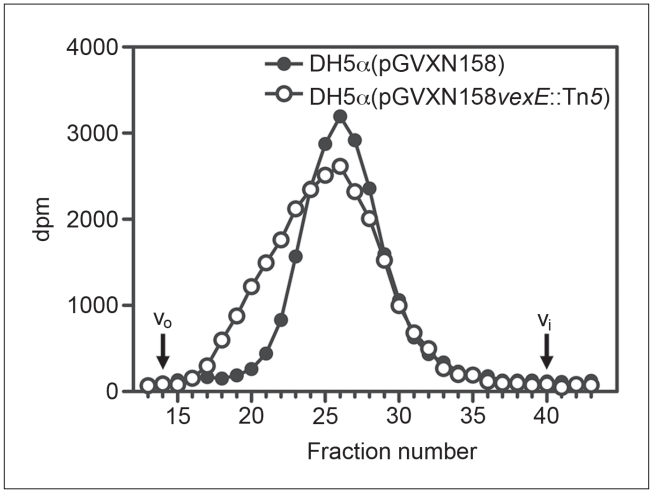

Figure 5: Size exclusion chromatography of exported Vi produced by wild type and *vexE* transposon insertion mutant.
DH5α(pGVXN158) and DH5α(pGVXN158*vexE*::Tn*5*) cells were metabolically labeled with [^{3}H]GlcNAc and the Vi polysaccharide was purified from the cell culture supernatants. Purified [^{3}H]Vi polysaccharide was analyzed by size exclusion chromatography using a Sephacryl S-1000 column. The elution profiles of the [^{3}H]Vi preparations are shown. The void volume (v_o) and the inclusion volume (v_i) of the column are indicated.

Table III
O-acetyl content of Vi.

Strain	O-acetyl content (mmol g^{-1})
S. Typhi BRD948	2.4 ± 0.2
DH5α(pGVXN158)	2.7 ± 0.2
DH5α(pGVXN158*vexC*::Tn*5*)	2.5 ± 0.3
DH5α(pGVXN158*vexE*::Tn*5*)	2.4 ± 0.2

transposon insertion mutant derivative could be due to a different degree of O-acetylation. We therefore determined O-acetyl contents of Vi produced by *S.* Typhi BRD948, *E. coli* DH5α(pGVXN158), DH5α(pGVXN158*vexC*::Tn*5*) or DH5α-(pGVXN158*vexE*::Tn*5*). Vi polysaccharide of these derivatives was purified either from the cell culture supernatant (in *S.* Typhi BRD948, DH5α(pGVXN158) and DH5α(pGVXN158*vexE*::-Tn*5*)) or from cell lysates (in DH5α(pGVXN158*vexC*::Tn*5*)). The O-acetyl content of the purified Vi preparations was then measured by the alkaline hydroxylamine method of Hestrin [136]. The average values of the O-acetyl content from three separate preparations are presented in **Table III**. The O-acetyl content was comparable in all different derivatives, being around 2.5 mmol per gram of dried Vi polysaccharide, which corresponds to a level of O-acetylation of around 60 %. These data show that the Vi produced in *E. coli* has a similar O-acetyl content as the Vi expressed by *S.* Typhi. The undistinguishable levels of O-acetylation of Vi purified from the cell lysate of DH5α(pGVXN158*vexC*::Tn*5*) indicates that the O-acetylation reaction takes place in the cytosol and that the reaction is independent of polymer translocation. Furthermore, the inability of DH5α(pGVXN158*vexE*::Tn*5*) to form a capsule does not correlate with different levels of O-acetylation of the Vi polymer.

Discussion

Typhoid fever remains a serious public health problem in many developing countries, with, according to conservative WHO estimates, 16 million cases occurring each year, including about 600,000 deaths. The Vi capsule plays an important role in *S.* Typhi virulence by increasing the infectivity of the pathogen and the severity of the disease. Parenteral vaccines based on purified Vi CPS have been licensed and are widely used. Yet, many aspects of the biosynthesis, export and capsule formation of this important polysaccharide remain unclear. Here, a detailed molecular characterization of the *viaB* operon, encoding the biosynthetic machinery for Vi capsule formation is reported.

O-acetylation of the Vi

The Vi CPS is a linear, acidic homopolymer of α-1,4-linked *N*-acetylgalactosaminuronate (D-GalNAcA), 60–90% O-acetylated at the C-3. The bulky nonpolar O-acetyl groups make up most of the surface of the Vi and complete O-deacetylation eliminates the immunogenicity of this CPS [35]. The bacterial enzyme capable of introducing this modification in the Vi has not yet been described. PFAM analysis of the proteins encoded in the *viaB* operon did not identify an O-acetyltransferase domain, indicating that the gene is located outside the *viaB* cluster in the *S.* Typhi genome. This situation has been demonstrated for the phase-variable O-acetylation of the group 2 capsule of *E. coli* K1. In this bacteria the modification is catalyzed by a prophage encoded O-acetyltransferase that is not linked to the capsular cluster [137].

Clearly the *viaB* operon can be functionally expressed in *E. coli* [122,138]. However, the Vi expressed in *E. coli* has not yet been thoroughly characterized and the presence of O-acetyl modifications was equivocal. We found that the Vi produced in *E. coli* is recognized by Vi phage types I–VII, thereby converting *E. coli* into a Vi phage susceptible bacteria. Recognition of Vi polymer by a Vi

phage is dependent on the presence of O-acetyl groups at the C-3 position of the *N*-acetylgalactosaminuronate (GalNAcA) building blocks and it has been shown that phage particles have deacetylating properties [135]. A conserved protein domain carrying an acetyl esterase was found to be associated with at least one tail fiber gene for all Vi phages [139]. This implies that in contrast to the tip enzymes of K1 or K5 phages, that degrade the polysaccharide backbone, the Vi phages specifically target the acetyl modification on the sugar polymer. Besides the observed susceptibility to Vi phage infection, we could show through chemical analysis that the Vi produced in *E. coli* is substantially O-acetylated. In addition, a similar degree of O-acetylation could be measured in an intracellular Vi preparation produced by a transporter deficient mutant (DH5α(pGVXN158*vexC*::Tn*5*)), indicating that O-acetylation is taking place in the cytoplasm and that it is a translocation independent process.

Vi productivity in *E. coli* DH5α(pGVXN158) is higher compared to *S.* Typhi BRD948

It was observed that *E. coli* cells expressing the wild-type *viaB* operon produce around 6–7 times more Vi than *S.* Typhi BRD948. This observation could be explained by the presence of the *viaB* cluster in different copy numbers in the particular expression systems. The pLAFR1 plasmid derivative that contains the *viaB* cluster is reported to be present in 5–7 copies per cell [140], whereas *S.* Typhi BRD948 holds a single *viaB* operon. Furthermore, spontaneous loss of SPI-7 in Vi-negative *S.* Typhi has been reported in stored isolates [141]. The robust plasmid borne expression of the Vi might additionally account for the difference seen in CPS productivity. The observed high efficiency of wild-type Vi polysaccharide production in *E. coli* makes this strain a good model system to characterize Vi capsule formation and an interesting Vi production system.

tviBCDE is the minimal set of genes required for Vi polysaccharide synthesis

Our data confirm the results obtained by Virlogeux *et al.*, 1995, that TviB, TviC, TviD and TviE are required for Vi polymer assembly. Firstly, no Vi could be detected in these individual transposon insertion mutants. Secondly, intracellular Vi was identified in *E. coli* cells carrying a plasmid encoding *tviBCDE* (results not shown). The latter result indicates that the Vi polymer is assembled in the cytoplasm and that assembly is independent of the translocation machinery. Similar results have been obtained with the well-characterized group 2 capsule of *E. coli* K1. It has been shown that cells harboring a plasmid encoding the biosynthetic region-2 genes alone can synthesize intracellular polysialic acid [142]. Additionally, synthesis of full-length polymer has been shown to occur *in vitro* and in a variety of transport-deficient backgrounds *in vivo* [143,144].

Synthesis and export of the Vi polysaccharide seem to be coupled processes

It appears to be common to group 2-like capsules that biosynthesis of intracellular polymer is independent of the sugar export machinery. However, Vi quantification in the different *vex*::Tn*5* transposon insertion mutants that encode the CPS transporter (*vexABCD*) by ELISA demonstrated different productivity in the individual mutants. Whereas DH5α(pGVXN158*vexA*::Tn*5*) expressed almost wild-type levels of Vi, the CPS productivity in DH5α(pGVXN158*vexB*::Tn*5*), DH5α(pGVXN158*vexC*::Tn*5*) and DH5α(pGVXN158*vexD*::Tn*5*) was greatly reduced. This result indicates that synthesis and export of the Vi are coupled processes. A reduction in endogenous glycosyltransferase activity has also been shown for *E. coli* K1 cells harboring export defects [128,143].

Moreover, the formation of a hetero-oligomeric complex consisting of both biosynthetic and export machinery has been demonstrated in *E. coli* K5 [145] and *E. coli* K1 [146]. It is possible that the predicted inner membrane associated components of the Vi transporter (VexB, VexC and VexD) interact with the cytoplasmic based biosynthetic machinery and that disruption of the transporter indirectly decreases biosynthesis of the Vi polymer by unknown mechanisms. It seems that this observed negative feedback mechanism between a non-functional exporter and the biosynthesis of Vi polymer does not exist when the outer membrane polysaccharide export protein VexA is nonfunctional. These mutant cells produce almost wild-type amounts of CPS. Furthermore, the Vi could sometimes be detected clustered against the inner membrane by transmission electron microscopy in DH5α(pGVXN158*vexA*::Tn*5*) cells, which might indicate the formation of a functional complex between inner membrane export and biosynthesis components. Nevertheless, periplasmic accumulations of Vi could not be detected in these mutant cells as it has been described for *E. coli* K1 and K5 mutations in the homologous gene *kpsD* [147,148].

DH5α(pGVXN158*vexC*::Tn*5*) cells accumulated the Vi polysaccharide in large intracellular, inclusion-like bodies as seen by transmission electron microscopy. This phenotype has also been described in *E. coli* K1 [144,149]. However, inactivation of the *E. coli* K1 ABC transporter protein KpsT, resulted in a slightly different localization of the K1 polymer within the cell, where it accumulated at discrete sites around the periphery of the cells, located against the cytosolic membrane.

High Vi expression and non-functional export machinery results in cell instability

Our data clearly demonstrate that the high levels of Vi produced in DH5α(pGVXN158*vexA*::Tn*5*) cells is not cell surface exposed. These cells can neither be agglutinated using Vi specific antisera nor be infected by a Vi phage. In addition, transmission electron microscopy pictures of these mutant cells do not show a capsule but intracellular accumulations of the Vi polymer. Substantial amounts of the cytosolic protein GroEL could be detected in the cell culture supernatant, indicating that large amounts of Vi produced by this export deficient mutant destabilize the cell. The Vi detected in the cell culture supernatant and in fixed cells by ELISA and immunoblot represents therefore not translocated but rather released polysaccharide from lysed cells. In transporter deficient mutants showing reduced Vi assembly activity (DH5α(pGVXN158*vexB*::Tn*5*), DH5α(pGVXN158*vexC*::Tn*5*) and DH5α(pGVXN158*vexD*::Tn*5*)) less GroEL was detected in the cell culture supernatant compared to the *vexA*::Tn*5* transposon insertion mutant. It seems that the level of Vi biosynthesis in transporter deficient mutants correlates with cell instability. The observed destabilizing effect of intracellular CPS accumulations on cell integrity also underlines the necessity that assembly and export have to be coupled and coordinated processes. However, the observed phenotype of a *vexA* mutant might be less pronounced in a *S*. Typhi where the Vi polysaccharide is not overexpressed.

Retention of the Vi polysaccharide on the cell surface is dependent on *VexE*

VexE shows no homologies to other proteins encoded in group 2-like capsular gene clusters and it is exclusively found in the *viaB* operon. PFAM analysis of VexE identified a C-terminal acyltransferase domain also found in HtrB (LpxL), an enzyme involved in Kdo_2-Lipid A biosynthesis. Vi quantification by ELISA demonstrated, that Vi production in DH5α(pGVXN158*vexE*::Tn*5*) cells was only slightly decreased compared to *E. coli* cells expressing the wild-type *viaB* operon. The Vi polysaccharide produced by a *vexE*::Tn*5* mutant is mainly found in the cell culture supernatant as determined by ELISA. There was no Vi visualized in the extracellular spaces by TEM and immunogold labeling, but this could be lost during sample preparation. Together, these results indicate that the Vi in a *vexE*::Tn*5* mutant is efficiently transported to the cell surface, but once translocated, the polysaccharide is not retained on the cell surface but is rather secreted. Therefore these mutant cells can neither be agglutinated using Vi specific antisera nor be infected by a Vi phage. Besides the observed inability of this Vi species to form a capsule, the polysaccharide also does not bind to nitrocellulose membranes, indicating that this species has different physical characteristics compared to the wild type Vi. We can rule out that the observed properties are due to a different degree in O-acetylation because in both Vi preparations a similar O-acetyl content was determined. However, these observations might be explained by a lipid modification that is introduced by the acyltransferase-like enzyme VexE. In addition, the polysaccharide produced by a *vexE*::Tn*5* mutant displays a broader size distribution compared to the wild-type Vi on a Sephacryl S-1000 column. This lipid modification might lead to a different stoke radius, explaining the different behavior in size exclusion chromatography. It is equivocal if the observed phenotype of a *vexE* mutant plays a biological role. It might be possible that *S.* Typhi can switch between a capsule producing and a Vi secreting state during differ-

ent stages of pathogenesis by regulating VexE activity. The precise function of VexE in Vi capsule formation should be addressed in future research.

Conclusions

In summary, we conclude that the Vi capsule biosynthesis shows many characteristics that are typical for group 2-like capsules. The Vi is completely assembled in the cytoplasm by the gene products of *tviBCDE*, and is subsequently transported to the cell surface by an ABC transporter encoded by *vexABCD*, that closely resembles the transporters of the *Haemophilus influenzae* type b and *Neisseria meningitides* group B CPSs. However, several genes indicative for *E. coli* group 2 capsular gene clusters, like *kpsFU* and *kpsSC*, that are believed to be involved in CMP-Kdo synthesis and the speculative addition of a diacylglycerophosphate-Kdo to the CPS [106], are absent in the *viaB* operon. In contrast, the *viaB* operon contains a gene, *vexE*, uniquely found in this cluster and possibly involved in lipidation of the Vi polysaccharide. Despite sharing the basic mechanism of group 2-like capsule formation, biosynthesis of the Vi capsule displays features uniquely found in this cell surface structure.

Materials and Methods

Strains, plasmids, and culture conditions

All bacterial strains and plasmids used in this study are listed in **Table IV**. Construction of the plasmids is described below. *E. coli* DH5α and EC100 were grown in LB medium (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter) or on LB agar (LB medium with the addition of 15 g agar per liter) at 37 °C. *S.* Typhi BRD948 [60] was grown in LB medium supplemented with 1 % v/v Aro-mix (40 mg L-phenylalanine, 40 mg L-tryptophan, 10 mg 4-aminobenzoic acid, and 10 mg 2,3-dihydroxybenzoic acid in 10 ml of ddH_2O) and 1 % v/v Tyr-mix (40 mg L-tyrosine disodium salt in 10 ml ddH_2O) at 37 °C. If appropriate, the media contained tetracycline (20 μg ml^{-1}), trimethoprim (100 μg ml^{-1}), or kanamycin (50 μg ml^{-1}). Cells containing pEXT22 derivatives were grown in the presence of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

DNA manipulations

Plasmid DNA was isolated using the NucleoSpin Plasmid or NucleoBond Xtra Maxi Plus kit (Macherey-Nagel). Total chromosomal DNA was isolated using NucleoSpin Tissue kit (Macherey-Nagel). Restriction enzymes (Fermentas), shrimp alkaline phosphatase (Fermentas), T4 DNA ligase (Fermentas), and Phusion High-Fidelity DNA polymerase (Finnzyme) were used according to the manufacturer's instructions. PCR and restriction fragments were purified for cloning using the NucleoSpin Extract II kit (Macherey-Nagel). All DNA sequencing was completed by Synergene Biotech GmbH (Switzerland) and synthetic oligonucleotides were ordered at Microsynth AG (Switzerland).

Table IV
Strains and plasmids used in this study.

Strain	Genotype or relevant description	Reference
S. Typhi BRD948	*S.* Typhi Ty2 Δ*aroC* Δ*aroD*	[60]
E. coli DH5α	K-12 φ80d*lacZ*Δ*M15 endA1 recA1 hsdR17*(rK–mK) *supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* F^-	Clonetech
E. coli EC100	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80d*lacZ*Δ*M15* Δ*lacX74 recA1 endA1 araD139* Δ(*ara, leu*)*7697 galU galK* λ^- *rpsL nupG*	Epicentre

Plasmid	Genotype or relevant description	Reference
pLAFR1	low copy-number broad host-range cosmid cloning vector; Tet^R	[140]
pGVXN157	pLAFR1 derivative with multiple cloning site inserted in *EcoR*I site	This study
pGVXN158	pGVXN157 derivative carrying *viaB* operon of *S.* Typhi BRD948 on an *Asc*I/*Spe*I fragment; Tet^R	This study
pGVXN209	pGVXN158 with *tviB*::Tn*5* insertion (pGVXN158*tviB*::Tn*5*); Tet^R, Tmp^R	This study
pGVXN224	pGVXN158 with *tviC*::Tn*5* insertion (pGVXN158*tviC*::Tn*5*); Tet^R, Tmp^R	This study
pGVXN198	pGVXN158 with *tviD*::Tn*5* insertion (pGVXN158*tviD*::Tn*5*); Tet^R, Tmp^R	This study
pGVXN200	pGVXN158 with *tviE*::Tn*5* insertion (pGVXN158*tviE*::Tn*5*); Tet^R, Tmp^R	This study
pGVXN225	pGVXN158 with *vexA*::Tn*5* insertion (pGVXN158*vexA*::Tn*5*); Tet^R, Tmp^R	This study
pGVXN227	pGVXN158 with *vexB*::Tn*5* insertion (pGVXN158*vexB*::Tn*5*); Tet^R, Tmp^R	This study
pGVXN201	pGVXN158 with *vexC*::Tn*5* insertion (pGVXN158*vexC*::Tn*5*); Tet^R, Tmp^R	This study
pGVXN202	pGVXN158 with *vexD*::Tn*5* insertion (pGVXN158*vexD*::Tn*5*); Tet^R, Tmp^R	This study
pGVXN204	pGVXN158 with *vexE*::Tn*5* insertion (pGVXN158*vexE*::Tn*5*); Tet^R, Tmp^R	This study

Plasmid constructions

pGVXN158 contains the *viaB* cluster cloned into the pLAFR1 [140] derivative pGVXN157. pGVXN157 contains a synthetic oligonucleotide cassette formed from annealing of 5'- AATTGGCGCGCCCGGGACTAGTCTTGGG and 5'- AATTCCCAAGACTAGTCCCGGGCGCGCC ligated into the *EcoR*I-digested pLAFR1 therefore introducing unique *Asc*I and *Spe*I single restriction sites. The *viaB* gene cluster was amplified from genomic DNA prepared from *S.* Typhi BRD948 using the primers 5'- AAAGGCGCGCCGGAGTATCAGTGTGGGGCATAATC and 5'- AAAACTAGTGGCCATGAGTCTGAAGCCAGGAGGAATT. The *viaB* cluster containing PCR fragment was inserted into the *Asc*I/*Spe*I digested pGVXN157 therefore producing pGVXN158.

Transposon insertion mutagenesis and screening of *viaB* insertion mutants

In vitro transposon insertion mutagenesis was done with pGVXN158 containing the *viaB* cluster using the EZ-Tn5 <DHFR-1> insertion kit (Epicentre Biotechnologies, Madison WI 53713, U.S.A.) according to manufacturer's instructions. The *in vitro* transposon insertion reaction was transformed into TransforMax EC100 electrocompetent *E. coli* (Epicentre) and insertion mutants were selected for the marker encoded by the EZ-Tn5 transposon (dihydrofolate reductase gene (*dhfr-1*)) on trimethoprim containing plates. To identify transposon insertions into single genes of the *viaB* cluster the library was screened for Vi negative colonies. Therefore a colony blot was performed. Transformants were transferred to a nitrocellulose membrane. After washing the membrane 3 x with PBST (PBS, 0.1 % Tween 20) for 10 min the membranes were blocked by incubating with 10 % milk in PBST for 1 h on a shaker at room temperature (RT). Membranes were incubated

with a monoclonal anti Vi antibody (P2B1G2/A9, [134]) diluted 1:100 in 1 % milk in PBST for 1 h. After washing the membranes 3 x with PBST the membranes were incubated with a horseradish peroxidase labeled goat anti-mouse IgG (Sigma, A9824) diluted 1:2000 in 1 % milk in PBST. Membranes were developed using 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system for membranes (Sigma). Plasmid DNA of Vi negative colonies or colonies that appeared to have a reduced expression of the Vi capsule was isolated and the transposon insertion site was mapped by sequencing out of the EZ-Tn5 <DHFR-1> transposon using primers DHFR-1 RP-1 and DHFR-1 FP-1 provided with the EZ-Tn5 transposon insertion kit. Single gene insertion mutants of every gene of the *viaB* cluster except *tviA* were selected. In all the selected mutants the coding strand of the transposon encoded *dhfr-1* gene was identical to the coding strand of the genes in the *viaB* cluster.

Antibody detection of Vi antigen on washed cells and in cell culture supernatants

Overnight cultures were centrifuged and the supernatant was separated from the cell pellet. The cells were washed 3 x with PBS and finally resuspended in an equal volume of PBS. 100 µl of cell culture supernatant and washed cells were pipetted onto nitrocellulose, and the membranes were blocked for 1 h at room temperature in 10 % milk in PBST (PBS, 0.1 % Tween 20). The presence of Vi was detected by using a monoclonal anti Vi antibody (P2B1G2/A9, [134]) as the primary antiserum, followed by horseradish peroxidase labeled goat anti-mouse IgG (Sigma, A9824). Membranes were developed using 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system for membranes (Sigma).

Sample preparation for measuring Vi content by ELISA

1 ml of an overnight culture was centrifuged 5 min at 20'000 g using an eppendorf microcentrifuge. The supernatant was separated from the cell pellet and used to measure Vi content. The pellet was washed 3 x with PBS and divided into two eppendorf tubes. One half of the cells was fixed by resuspending the pellet in 4 % paraformaldehyde in PBS and incubation for 30 min at RT. Afterwards the fixed cells were washed 3 x with PBS and cell associated Vi was measured. The other half of the washed cells was lysed using B-PER protein extraction reagents (Pierce) according to manufacturer's instructions. Protein and Vi contents were measured in the cell lysate by the bicinchoninic acid assay (BCA, Pierce) and ELISA. The amount of intracellular Vi was defined using the difference in Vi measured in cell lysates and in fixed cells. The Vi content of different samples was normalized to the protein amount measured in the cell pellets.

Vi quantification by ELISA

Vi polysaccharide content of samples was measured using a sandwich ELISA. Two monoclonal Vi specific mouse antibodies P2B1G2/A9 (IgG1, coating antibody) and P5B2D8/A9 (IgM, primary antibody) were used in the ELISA and are described elsewhere [134]. In order to saturate the binding of the Vi to the ELISA plate, the coating antibody P2B1G2/A9 was partially purified from the hybridoma supernatant using a 5 ml HiTrap Protein A HP column (GE Healthcare). Briefly, 50 ml of P2B1G2/A9 hybridoma supernatant were diluted 1:10 with binding buffer (20 mM sodium phosphate, pH 7.5) and loaded on the Protein A column using a peristaltic pump at a flow rate of 20 ml min^{-1}. The column was eluted with 100 mM citric acid pH 3.25 and 1 ml fractions were collected. The tubes destined to collect the antibody fractions contained 100 µl 1 M Tris-HCl, pH 9.0 to instantly neutralize the sample.

Flat bottom 96 well micro-titer plates (Nunc immuno MaxiSorb) were coated with 50 µl of the partially purified antibody P2B1G2/A9, diluted 1:100 in PBS, at 4 °C overnight. The coating solution was poured away and the plate was submerged and vigorously agitated in 4000 ml of wash buffer (1 x PBS with 0.05 % Triton X 100). This washing step was performed at least 4 times to completely wash out unbound antibody. Subsequently, the plate was dried by spinning upside down in a micro plate rotor. This washing procedure was always applied in further washing steps. Each well was completely filled with 300 µl of blocking buffer (1 x PBS with 2.5 % BSA (globulin free BSA, Sigma, A7030)) and incubated 2 h at RT on a plate shaker. After washing and drying the plate the sample was applied in an appropriate dilution in dilution buffer (1 x PBS with 0.5 % BSA). As a standard, Vi polysaccharide (Typhim Vi, Sanofi Pasteur MSD) was used in several dilutions in the range from 0–4 ng ml^{-1}. 100 µl of sample and standard were applied in doublicates to the corresponding wells and incubated 1 h at RT on a plate shaker. After washing and drying the plate, 100 µl of the primary antibody (hybridoma supernatant of P5B2D8/A9) diluted 1:100 in dilution buffer was added to the wells and incubated 1 h at RT on a plate shaker. After another washing and drying step the wells were incubated with 100 µl of secondary antibody (goat anti-mouse IgM peroxidase conjugated, Sigma A8786) diluted 1:2000 in dilution buffer for 1 h at RT on a plate shaker. Following washing and drying the reaction was developed with 100 µl of Ultra TMB substrate (3,3',5,5'-tetramethybenzidine liquid substrate, Pierce) for 15 min and stopped with the addition 100 µl of 2 M sulphuric acid. The bottom surface of the plate was wiped with 70 % 2-propanol and the absorption at 450 nm was measured.

Vi polysaccharide purification

Vi polysaccharide was purified by a modified procedure as described elsewhere [150]. The Vi was either purified from the cell culture supernatant (in strains *S.* Typhi BRD948, DH5α(pGVXN158) and DH5α(pGVXN158*vexE*::Tn*5*)) or from sonicated cells that were treated with proteinase K and nucleases (in strain DH5α-(pGVXN158*vexC*::Tn*5*)). Vi was precipitated from the supernatant or enzyme treated cell lysate with 0.1% hexadecyltrimethylammonium bromide (CTAB, Sigma, H6269). 20 g l^{-1} celite 545 (Sigma, 20199-U) was added and the mixture was stirred for 1 h at RT in order to allow the formation of a polysaccharide-CTAB complex, which adsorbs onto the celite. The celite was poured into a reservoir of appropriate size (Extract-clean EV SPE Reservoir, Socochim S.A.) equipped with a frit (Socochim S.A.). The column was washed successively by gravity flow with 10 column volumes (CV) of 0.05% CTAB, 10 CV of 20% ethanol, 50 mM sodium phosphate buffer pH 6.0, and 15 CV of 45% ethanol to eliminate adsorbed impurities. The Vi polysaccharide was finally eluted with 1.5 CV of 50% ethanol, 0.4 M NaCl. Following elution, the polysaccharide was precipitated by addition of ethanol to a final concentration of 80% and incubation for 20 min at RT. The precipitate was washed twice with 80% ethanol prior to lyophilization. The protein and nucleic acid content of the purified Vi polysaccharide was determined by the bicinchoninic acid assay (BCA, Pierce) and UV spectroscopy respectively.

Determination of O-acetyl content in the Vi polysaccharide

The method was used as described elsewhere [136]. Briefly, the O-acetyl groups were released by mild base treatment of the Vi polysaccharide solution. The O-acetyl groups reacted with the added hydroxylamine in alkali to form hydroxamic acid. The formed hydroxamic acid was measured by the formation of a purple-brown complex with Fe^{3+}. Acetylcholine was used as a standard.

Radiolabeling of Vi polysaccharide

Cells were cultured in LB medium supplemented with antibiotics at 37 °C in the shaker incubator (180 rpm). Cultures were inoculated from an overnight culture to an A_{600} of 0.01. After 4 cell doublings, 1 μCi ml^{-1} [^{3}H]GlcNAc (American Radiolabeled Chemicals, Inc., ART 0142) was added and the culture was incubated 2 h at 37 °C in the shaker incubator. After incubation the cells were centrifuged and the cell culture supernatant was used to purify the labeled Vi polysaccharide as described above. After drying of the purified Vi, the polysaccharide was resuspended in 50 mM NaCl. Incorporation of radioactivity in the purified samples was measured and normalized to the protein content measured in the cell pellets.

Size exclusion chromatography

Sephacryl S-1000 (GE Lifescience) gel filtration medium was used in a column with the proportions of 23 cm x 1.7 cm (height x diameter). Chromatography was done by gravity flow using 50 mM NaCl as the mobile phase and 1 ml fractions were collected.

Transmission Electron Microscopy (TEM)

TEM was done as described elsewhere [151].

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Engineering, conjugation, and immunogenicity assessment of *Escherichia coli* O121 O-antigen for its potential use as a typhoid vaccine component

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Contributions

Cloning and expression of O121/Δ*wbqG* O antigen locus
LPS analysis
Glycoconjugate production and purification
Purification of O121 LPS
Purification of Vi and modification with tyramine
ELISA

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Abstract

State-of-the-art production technologies for conjugate vaccines are complex, multi-step processes. An alternative approach to produce glycoconjugates is based on the bacterial N-linked protein glycosylation system first described in *Campylobacter jejuni*. The *C. jejuni* N-glycosylation system has been successfully transferred into *Escherichia coli,* enabling *in vivo* production of customized recombinant glycoproteins. However, some antigenic bacterial cell surface polysaccharides, like the Vi antigen of *Salmonella enterica* serovar Typhi, have not been reported to be accessible to the bacterial oligosaccharyltransferase PglB, hence hamper development of novel conjugate vaccines against typhoid fever. In this report, Vi-like polysaccharide structures that can be transferred by PglB were evaluated as typhoid vaccine components. A polysaccharide fulfilling these requirements was found in *Escherichia coli* serovar O121. Inactivation of the *E. coli* O121 O antigen cluster encoded gene *wbqG* resulted in expression of O polysaccharides reactive with antibodies raised against the Vi antigen. The structure of the recombinantly expressed mutant O polysaccharide was elucidated using a novel HPLC and mass spectrometry based method for purified undecaprenyl pyrophosphate (Und-PP)-linked glycans, and the presence of epitopes also found in the Vi antigen was confirmed. The mutant O antigen structure was transferred to acceptor proteins using the bacterial N-glycosylation system, and immunogenicity of the resulting conjugates was evaluated in mice. The conjugate-induced antibodies reacted in an enzyme-linked immunosorbent assay with *E. coli* O121 LPS. One animal developed a significant rise in serum immunoglobulin anti-Vi titer upon immunization.

Introduction

Typhoid fever remains a serious public health problem of which there are 22–33 million cases occurring each year, including about 216'000–500'000 deaths [23]. The causative agent of this human systemic infection, *Salmonella enterica* subspecies I serovar Typhi (*S.* Typhi), is feco-orally transmitted through contaminated water and food. Hence, typhoid fever is endemic in less developed areas where sanitary conditions remain poor. This includes many countries of Asia, Africa and South America, where schoolchildren and young adults are most frequently affected [152]. Antimicrobial treatment of typhoid fever has become increasingly complicated through the emergence of multidrug resistant strains of *S.* Typhi [30].

Vaccination of high-risk populations is considered the most promising strategy for the control and prevention of typhoid fever. Currently, there are two licensed typhoid vaccines: the orally administered, live attenuated whole cell vaccine Ty21a and the purified Vi polysaccharide parenteral vaccine. The Ty21a vaccine has several disadvantages: (i) The mutations contributing to the attenuated phenotype of this *S.* Typhi strain are not fully defined [47], (ii) attenuated strains could theoretically revert to virulence and (iii) Ty21a is only modestly immunogenic and requires three to four initial doses and boosters every 5 years [57,58,153-155]. The usefulness of the Vi polysaccharide vaccine is limited by its age-related immunogenicity and the fact that immune responses against polysaccharides are T cell independent. Therefore, immunological memory cannot be established and revaccination does not elicit any booster response [156,157]. Due to these drawbacks, the replacement of current typhoid vaccines with well defined, well tolerated and highly immunogenic vaccines is desirable.

The disadvantages of a polysaccharide vaccine can be overcome by conjugating the carbohydrate to a protein carrier (conjugate vaccine). Upon conjugation, the polysaccharide behaves like a T cell dependent antigen. It has been shown that purified Vi polysaccharide covalently linked to recombinant *Pseudomonas aer-*

uginosa exotoxin A (EPA) induces a protective immune response against *S.* Typhi in young children [41,80].

However, production of conjugate vaccines is a complex, multi-step process. First, separate bacterial strains producing the recombinant protein carrier and the polysaccharide antigen have to be cultivated. The polysaccharide and the protein carrier have to be purified by different procedures, before the two components are chemically coupled. The last step involves additional purification steps for obtaining the final product. This laborious production process has disadvantages: (i) several purification steps are required, where considerable losses might occur and (ii) due to the random nature of chemical coupling the product is not a uniform structure but a mixture of different glycoconjugates, with potentially different efficacy profile.

A novel approach to produce conjugate vaccines is based on the bacterial N-linked glycosylation system first described in *Campylobacter jejuni* [100,101]. This protein glycosylation system can be functionally transferred into *Escherichia coli* to produce specified glycoproteins *in vivo* [101]. It has been shown that various bacterial polysaccharide structures assembled on the lipid carrier undecaprenyl pyrophosphate (Und-PP) can be transferred to selected periplasmic proteins by the oligosaccharyltransferase PglB of *C. jejuni* [102,103]. Furthermore, it is possible to produce recombinant glycoproteins by grafting the consensus sequence for N-glycosylation (D/E-Y-N-X-S/T, where Y and X can be any amino acid except proline) into proteins which are otherwise not glycosylated [104]. This cost-efficient *in vivo* production of glycoconjugates represents an alternative to the conventional manufacturing process.

Vi is a linear, acidic homopolymer of α-1,4-linked N-acetylgalactosaminuronic acid (D-GalNAcA) residues, 60-90 % O-acetylated at C-3 **(Figure 1)**. Immunogenicity of the Vi is closely related to its degree of O-acetylation [35,36]. Analysis of the bioinformatics signature of the Vi biosynthetic gene cluster *(viaB)* highlighted the presence of a putative ABC transporter and the absence of a *wzy* polymerase. Therefore, biosynthesis of Vi is thought to be

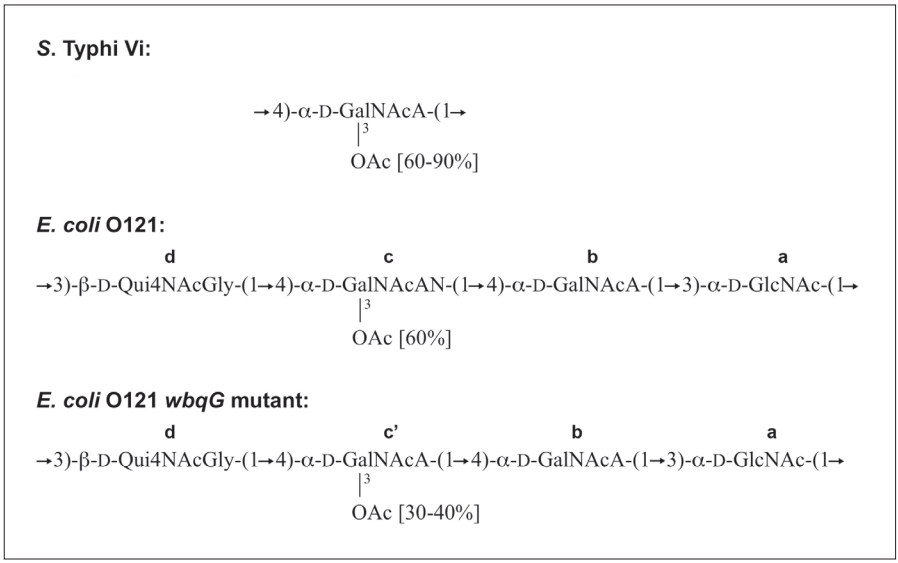


Figure 1: Structure of the *Salmonella* Typhi Vi polysaccharide and the repeating unit of the *Escherichia coli* O121 O antigen. Mutation of the O121 O antigen cluster encoded gene *wbqG* results in expression of a modified O polysaccharide structure. GalNAcA: 2-acetamido-2-deoxy-D-galacturonic acid; GalNAcAN: 2-acetamido-2-deoxy-D-galacturonamide; Qui4N: 4-amino-4,6-dideoxy-D-glucose.

similar to group 2 capsular polysaccharides (CPS). This class of CPSs is most likely not assembled on Und-PP and no periplasmic intermediates exist [106], which are both prerequisites for being a substrate of PglB. However, Und-PP-linked polysaccharides exist in bacteria that resemble the Vi antigen. The O antigen of *E. coli* O121 (identical to *Shigella dysenteriae* type 7) contains an N-acetylgalactosaminuronamide (D-GalNAcAN) residue, partially O-acetylated at the C-3, which is α-1,4-linked to a D-GalNAcA **(Figure 1)** [158]. The O antigen cluster of *E. coli* O121 contains the gene *wbqG*, encoding a glutamine-dependent amidotransferase involved in the biosynthesis of UDP-D-GalNAcAN [159]. It has

been shown that the O antigen of an *E. coli* O121 *wbqG* mutant contains D-GalNAcA (30-40 % O-acetylated at C-3) instead of D-GalNAcAN [159]. Hence, this mutant O polysaccharide contains structural similarities to the Vi **(Figure 1)**.

In this study the possibilities of producing a typhoid conjugate vaccine using the bacterial N-glycosylation system were exploited. The cross-reactivity of the *E. coli* O121 *wbqG* mutant O antigen with antibodies raised against the Vi is reported. Furthermore, glycoconjugates were produced and their immunogenicity was analyzed in mice.

Results

Analysis of the *E. coli* O121 *wbqG* mutant O polysaccharide.

First we examined whether the O polysaccharide produced by an *E. coli* O121 *wbqG* mutant would be recognized by antibodies specific for the *Salmonella* Typhi Vi capsular polysaccharide. Therefore, the *E. coli* O121 O antigen gene cluster was cloned, and the open reading frame of *wbqG* was interrupted by insertion of a STOP codon containing oligocassette. The cloned plasmids were transformed into the *E. coli* K-12 strain W3110 and the lipopolysaccharide (LPS) was analyzed by SDS-PAGE and staining with silver, or after transferring to a nitrocellulose membrane by Western blot **(Figure 2A)**. As previously reported, mutation of the *wbqG* gene did not abolish O antigen expression [159]. However, the LPS profile of the *wbqG* mutant visualized in the silver-stained polyacrylamide gel differed from the wild type in several points: (i) the staining of polymerized O antigen containing bands is fainter relative to wild type LPS, (ii) the band consisting of one O antigen repeating unit attached to the lipid A-core (core+1RU) stained more intensely and (iii) the O antigen containing bands migrated faster than the equivalent bands of the wild type LPS. It was estimated that both LPS profiles contained an average of 12 O antigen

repeat units attached to the lipid A-core by analyzing an overexposed silver-stained SDS-PAGE gel. Western blot analysis of the LPS revealed that anti-O121 sera reacted with the *wbqG* mutant O antigen. Interestingly, the *wbqG* mutant O polysaccharide was recognized by anti-Vi serum.

In order to confirm the structure of the expressed O antigen repeat unit and to determine the degree of O-acetylation, glycolipids were extracted from *E. coli* SCM6 strains expressing either the O121 wild type or the *wbqG* mutant O antigen. The lipid-linked oligosaccharides were purified using a C_{18} SepPak column and treatment with mild acid specifically released Und-PP-linked glycans. After an additional purification step using again a C_{18} SepPak column, the glycans were labeled with 2-aminobenzamide (2AB) and subsequently resolved by normal phase HPLC using a GlycoSep N column. **Figure 2B** shows a section of the chromatogram where single repeat units and short polymerized O antigens are expected to elute. Fractions containing putative 2AB-labeled glycan species were analyzed by mass spectrometry (MS) **(Figure 3)**, and the glycan structures identified by MS are illustrated in **Figure 2B**.

The chromatogram of the 2AB-labeled glycans prepared from SCM6 cells expressing the O121 wild type O antigen, featured a peak eluting at 58.8 min. In this peak fraction a molecule with a mass-to-charge ratio (m/z) of 1083 was identified. The peak fraction with the retention time of 65.1 min contained mainly a species with m/z of 1041. This detected m/z corresponded to the single-charged sodium adduct of a 2AB-labeled, non-acetylated O121 wild type subunit. The difference between the two detected masses corresponded to 42 Da, which is the mass difference between an O-acetyl and a hydroxyl group. These two species were subjected to collisionally induced dissociation (CID) MS-MS. The series of single-charged fragment ions obtained from the precursor with m/z of 1083 **(Figure 3A)** was consistent with glycosidic cleavage products from the 2AB-labeled O121 wild type O antigen repeat unit, containing an O-acetyl group at residue c. Whereas the CID MS-MS spectra of the molecular species with m/z of 1041 corresponded to the non-acetylated 2AB-labeled O121 subunit **(Figure 3B)**.

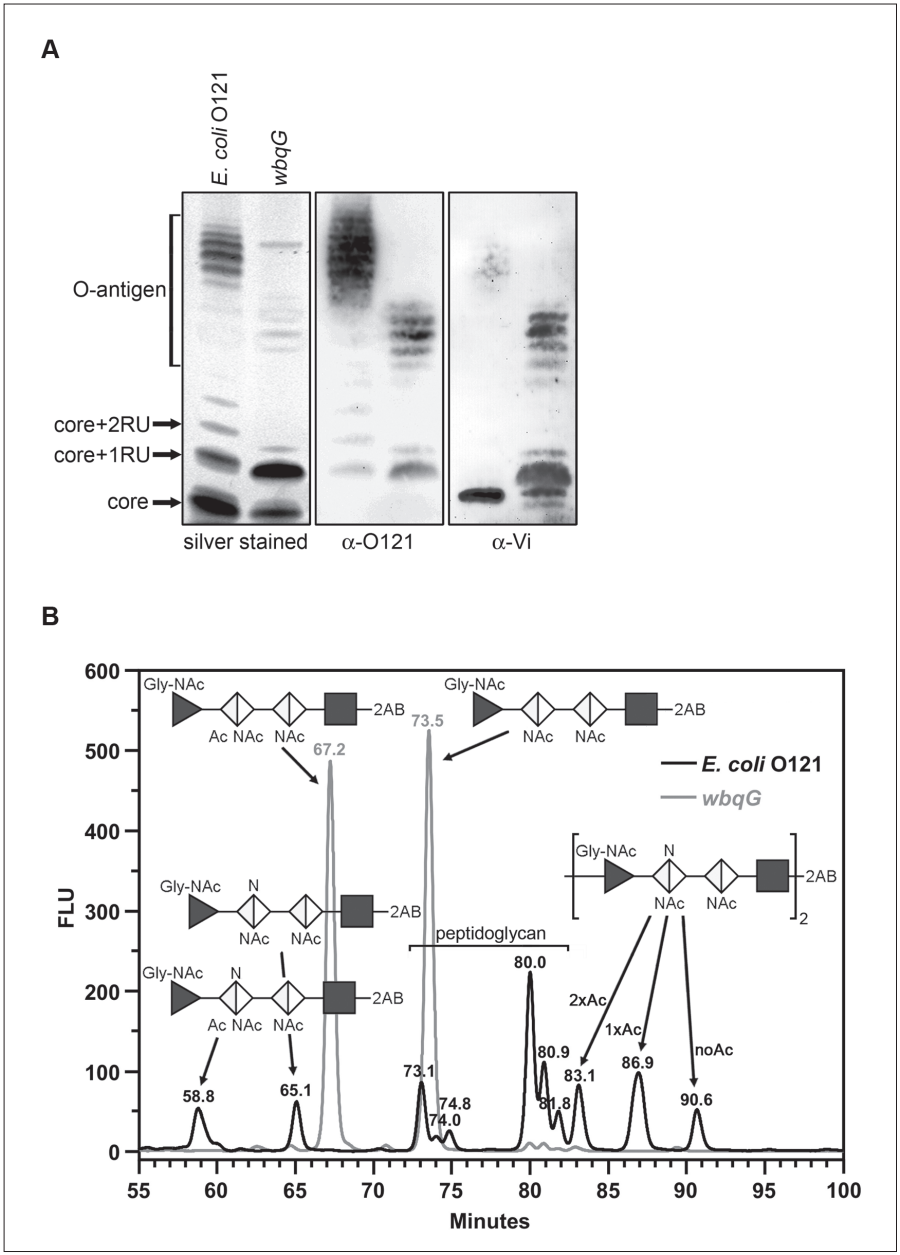

A
E. coli O121
wbqG
O-antigen
core+2RU
core+1RU
core
silver stained
α-O121
α-Vi
B
FLU
Minutes
E. coli O121
wbqG
Gly-NAc
2AB
peptidoglycan
67.2
73.5
58.8
65.1
73.1
74.8
74.0
80.0
80.9
81.8
83.1
86.9
90.6
2xAc
1xAc
noAc

Figure 2: O polysaccharide analysis of *E. coli* O121 and its *wbqG* mutant derivative.

A LPS from *E. coli* W3110 cells expressing the O121 wild type O antigen gene cluster or its *wbqG* mutant derivative was separated by SDS-PAGE and stained with silver or after transfer to a nitrocellulose membrane detected with anti-O121 and anti-Vi antibodies. Mutation of *wbqG* results in the assembly of a modified O antigen reactive with anti-Vi sera.

B Und-PP-linked glycans were extracted from *E. coli* SCM6 cells expressing the O121 wild type O antigen gene cluster or its *wbqG* mutant derivative followed by 2AB labeling and separation by normal phase HPLC using a GlycoSep N column. Individual peak fractions were analyzed by mass spectrometry and the identified glycan structures are indicated. (black square): N-acetylhexosamine; (black right-pointing triangle): dideoxyhexosamine; (white diamond): hexuronic acid; (white diamond)^{N}: hexuronamide; Ac: acetyl; NAc: N-acetyl.

The chromatogram of the 2AB-labeled glycans prepared from SCM6 cells expressing the *wbqG* mutant polysaccharide revealed two prominent peaks. In the peak fraction with the retention time of 67.2 min a molecule with m/z of 1084 was detected. This measured mass differed by 1 Da from the mass measured in the corresponding peak of the O121 wild type trace eluting at 58.8 min. Likewise an m/z of 1042 was measured for the 2AB-labeled molecule with a retention time of 73.5 min. CID MS-MS of these precursor ions **(Figure 3C and 3D)** resulted in a fragmentation pattern that resembled the spectra obtained from the O121 wild type 2AB-labeled glycans. The measured mass difference of 1 Da was assigned to residue c of the glycan structure. The mass difference of 1 Da corresponded to the calculated mass difference between an acid and an amide group, in agreement with the published structure of the *wbqG* mutant O antigen [159].

Furthermore polymerized 2AB-labeled O antigen subunits were identified in the O121 wild type trace. Two subunits variably O-acetylated were identified in the peak fractions with the retention times 83.1 min. 86.9 min and 90.6 min respectively. The dou-

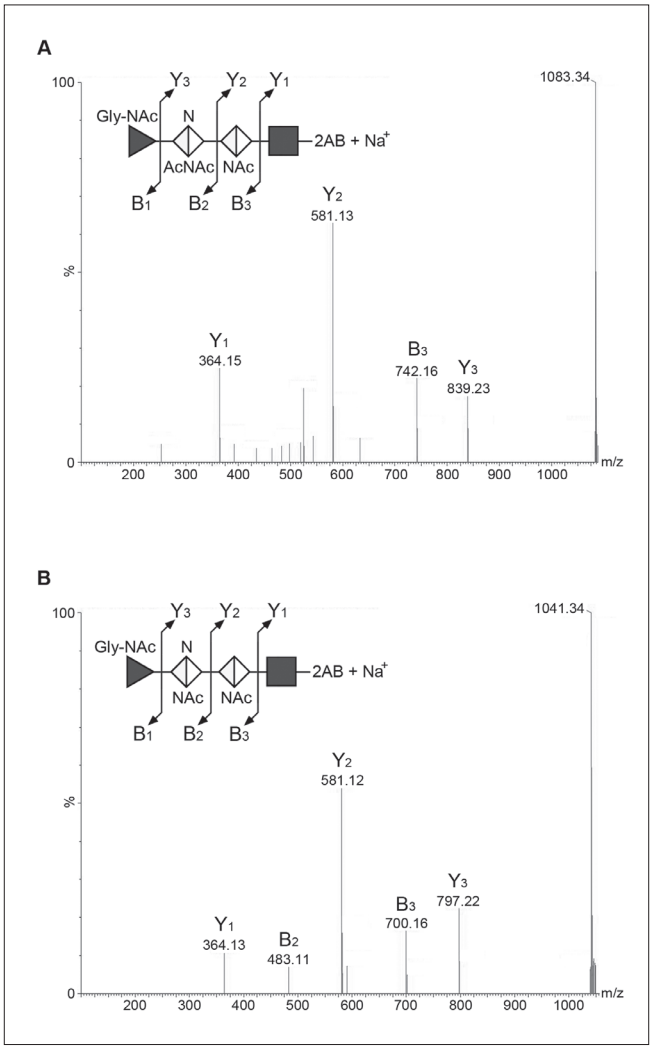
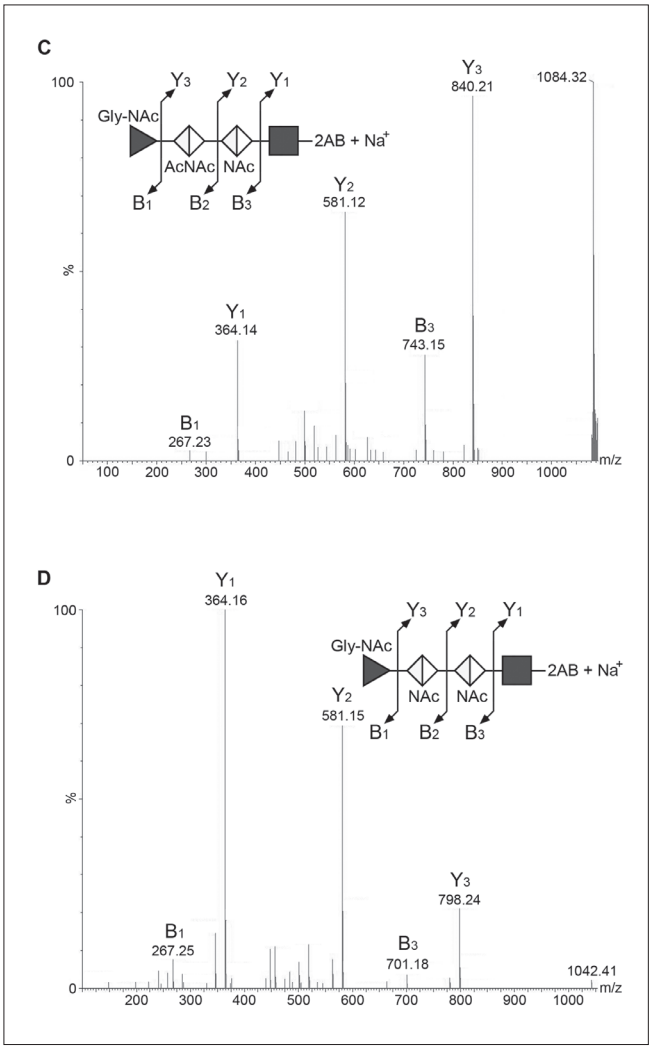


Figure 3: CID MS-MS spectra of glycan species separated by normal phase HPLC.

The CID MS-MS spectra correspond to the glycan species identified in the individual peak fractions seen in Figure 2B with the retention times: **A** 58.8 min. **B** 65.1 min. **C** 67.2 min. **D** 73.5 min.


C
100
%
0
Y3
Y2
Y1
Gly-NAc
AcNAc
NAc
2AB + Na+
B1
B2
B3
Y3
840.21
1084.32
Y2
581.12
Y1
364.14
B3
743.15
B1
267.23
100 200 300 400 500 600 700 800 900 1000
m/z
D
100
%
0
Y1
364.16
Y3
Y2
Y1
Gly-NAc
NAc
NAc
2AB + Na+
Y2
581.15
B1
B2
B3
Y3
798.24
B1
267.25
B3
701.18
1042.41
200 300 400 500 600 700 800 900 1000
m/z

ble acetylated species eluted first followed by the single acetylated and non-acetylated form. Due to the separation of the acetylated and non-acetylated forms, the degree of O-acetylation could be determined. In both strains approximately 50% of the single repeating units were O-acetylated.

Precursors of the peptidoglycan monomer were also identified in some peaks of the O121 wild type trace **(Figure 2B)**. Peptidoglycan precursors are also assembled on undecaprenyl pyrophosphate and are expected to be purified and labeled with the method used for O antigen subunits.

Production of glycoconjugates

The structure of the O121 *wbqG* mutant was confirmed, and it was shown to be cross-reactive with antibodies raised against the Vi antigen. Next, we examined whether the *wbqG* mutant O polysaccharide can elicit antibodies that bind to the Vi. Therefore, glycoconjugates were prepared for immunization studies. Glycoproteins were produced by expressing the bacterial oligosaccharyltransferase PglB, the engineered periplasmic carrier protein EPA (toxoid recombinant *Pseudomonas aeruginosa* exotoxin A), and either the *E. coli* O121 wild type or the *wbqG* mutant antigen in the *E. coli* K12 derivative CLM24 [102]. Strain CLM24 lacks the O antigen ligase (WaaL). Therefore, the transfer of O antigen to lipid A-core is blocked and the Und-PP-linked O antigen substrate accumulates at the periplasmic face of the inner membrane providing the O antigen donor for the PglB-catalyzed transfer to specific asparagine residues within the protein acceptor. Additionally, *E. coli* K12 derivatives lack a functional endogenous O antigen gene cluster [160,161]. A plasmid encoded O antigen gene cluster can therefore be expressed without producing mixed O antigen populations.

As described elsewhere EPA was used as protein acceptor with a N-terminal signal sequence for Sec-dependent secretion to the periplasm, and a C-terminal hexahistidine tag for purification by affinity chromatography [105]. Furthermore, EPA contained two en-

gineered N-glycosylation sites. The low copy plasmid pGVXN114 was used for the expression of PglB under the control of the IPTG inducible *tac* promoter.

After induction of PglB and EPA, the newly synthesized glycoprotein was purified from periplasmic extracts by nickel affinity chromatography. Due to the presence of negatively charged polysaccharides in the glycoconjugate, anion exchange chromatography was used to separate the glycosylated from the non-glycosylated forms. Based on the separation of the two species it was found that in cultures expressing the O121 wild type O polysaccharide gene cluster approximately 70% of the total EPA was glycosylated. The glycosylation efficiency was lower in cultures expressing the *wbqG* mutant O antigen whereas 35% of the total carrier protein contained the glycan modification.

The purified glycoconjugates were separated by SDS-PAGE and visualized by Coomassie blue staining or by western blot after transfer to a nitrocellulose mambrane using anti-EPA, anti-O121, and anti-Vi antibodies **(Figure 4)**. By Coomassie blue staining a band of the same mass as that of unglycosylated EPA (70 kDa) could be detected in the purified O121 polysaccharide-EPA conjugate (O121-EPA), that is also recognized by the anti-EPA but not the anti-O121 sera. Therefore, unglycosylated EPA was largely removed in the glycoconjugate preparations. Mainly, a ladder of bands clustered between 100 and 130 kDa was detected by Coomassie blue staining. These bands reacted with anti-EPA serum, indicating modified forms of EPA. These larger polypeptides, but not EPA modified with the *Shigella dysenteriae* O1 antigen (O1-EPA) (described in [105]), were also detected with anti-O121 specific antibodies indicating the modification of the carrier with the co-expressed polysaccharide. EPA glycosylated with the *wbqG* mutant O polysaccharide (O121*wbqG*-EPA) was additionally stained with anti-Vi antibodies.

As determined by SDS-PAGE analysis, mainly mono-glycosylated EPA was purified, *i.e.* EPA modified on one of the two engineered glycosylation sites with the corresponding O-polysaccharide. Traces of di-glycosylated EPA could be detected by western blot in the purified O121*wbqG*-EPA sample **(Figure 4)**. The di-glycosylated

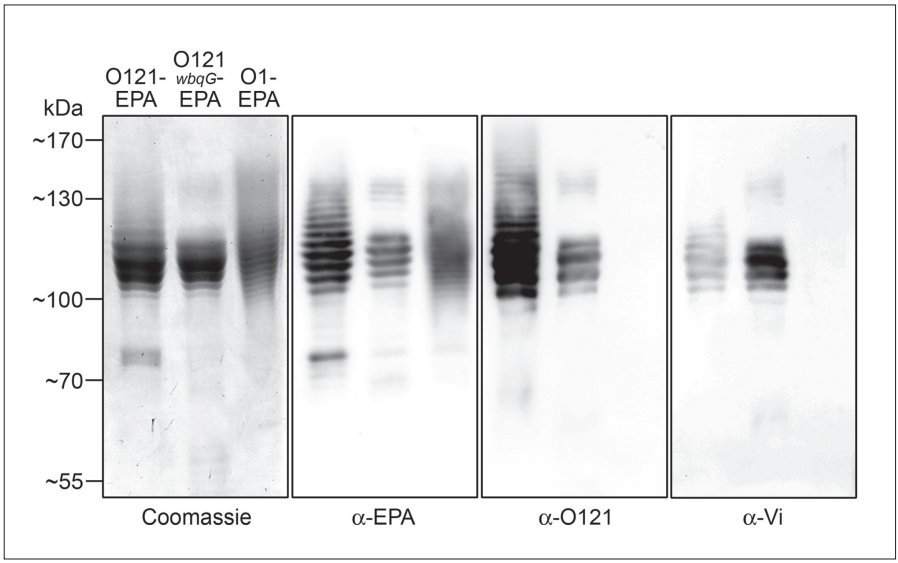


Figure 4: Production of glycoconjugates using the bacterial N-glycosylation system.
Glycoconjugates were produced in *E. coli* CLM24 by co-expressing the bacterial oligosaccharyltransferase PglB, the engineered carrier protein EPA, and genes driving the synthesis of an antigenic polysaccharide (*E. coli* O121, *E. coli* O121 *wbqG* mutant, *Shigella dysenteriae* O1). Purified glycoconjugates were analyzed by SDS-PAGE, followed by Coomassie blue staining or by western blot after transfer to nitrocellulose membranes using anti-EPA, anti-O121, and anti-Vi antibodies.

form of EPA runs as a second fainter ladder of bands slightly bigger than 130 kDa. As seen in Figure 2A, the expressed O-antigens display a modal chain length distribution with an average of 12 repeating units. Assuming the purified glycoconjugates consisted of mono-glycosylated EPA, containing a single polysaccharide chain of an average length of 12 repeating units, the sugar-to-protein weight ratio was estimated to be 0.15:1.

Immunogenicity of the glycoconjugates in mice and evaluation of the polysaccharide specific antibody response.

Next we analyzed the immune response elicited in mice upon immunization with the conjugate vaccines. Pilot experiments were conducted in small groups of CB6F1 mice to determine the dose range and adjuvantation of the purified glycoconjugates. These established that 20 μg of protein (approximately 3 μg of polysaccharide), in combination with Alum, were reproducibly immunogenic (data not shown). Subsequently, groups of CB6F1 mice (7 per group) were immunized subcutaneously on days 1, 22 and 57 with O121-EPA, O121*wbqG*-EPA, or with 5 μg of purified Vi polysaccharide (Typhim Vi, Sanofi Pasteur MSD). Mice were sample bled on days 32 and 67 and the sera were tested for the presence of anti-O121 LPS and anti-Vi total immunoglobulin (Ig). By day 67, a significant rise in serum Ig anti-O121 LPS titer was observed in 13 of 14 animals immunized with either conjugate (Figure 5A). One animal in the group of mice that were immunized with O121*wbqG*-EPA did not show seroconvertion. Interestingly, the same animal developed a significant rise in serum Ig anti-Vi titer (Figure 5B). As expected, the control group that was immunized with purified Vi polysaccharide did not show a detectable anti-O121 LPS response but a significant rise in serum Ig anti-Vi titer.

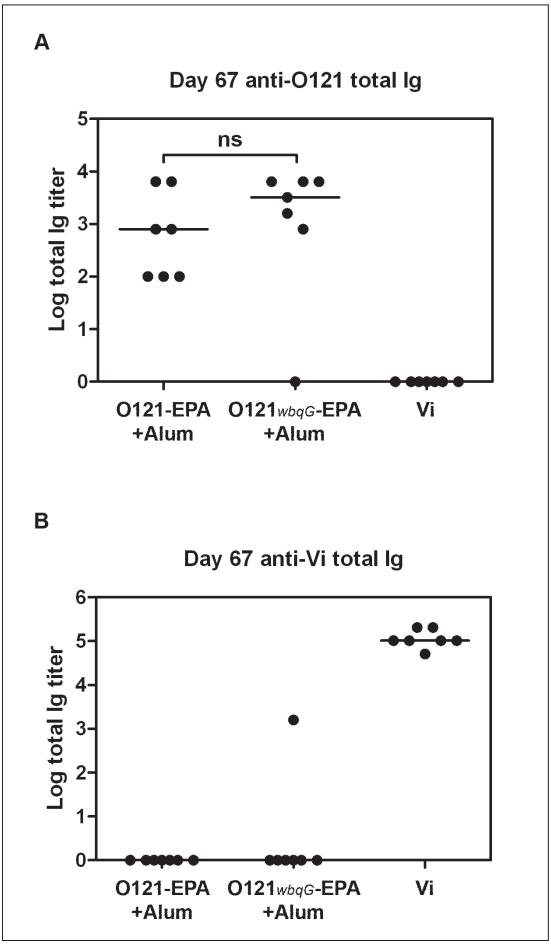


Figure 5: Immunization studies with glycoconjugates.
Groups of mice were immunized with purified glycoconjugates adjuvanted with Alum. The control group was immunized with purified Vi polysaccharide.

A Anti-O121 total immunoglobulin titers of sera collected on day 67.
B Anti-Vi antibody titers of sera collected on day 67. Data is represented as individual (black circle) and mean (-) titers. One animal immunized with the O121*wbqG*-EPA conjugate did not develop an O121-LPS specific antibody response, but the same animal showed a significant rise in anti-Vi antibody titer.

Discussion

In this study a novel method for the analysis of undecaprenyl pyrophosphate (Und-PP)-linked glycans is presented. The procedure described here is based on the method used to analyze dolichyl pyrophosphate (Dol-PP)-linked oligosaccharides of eukaryotic cells. Main modifications include an optimized extraction procedure for bacterial glycolipids and a purification step prior to glycan release by mild acid hydrolysis. The purification strategy of bacterial Und-PP-linked glycans is further complicated by the vast variety of different sugar structures assembled on this lipid carrier. The choice of an appropriate expression strain used to analyze a specific subclass of Und-PP-linked glycans is crucial. In this report, Und-PP-linked O polysaccharides were analyzed. Since Und-PP-linked O antigens represent an intermediate species of LPS biosynthesis, an *E. coli* strain was used lacking the O antigen ligase (Δ*waaL*). Therefore, Und-PP-linked O polysaccharides are not transferred to lipid A-core, resulting in accumulations of this lipid intermediate. If O antigens were expressed in a *waaL* positive strain no 2AB-labeled O glycans could be identified, most likely due to the rapid turnover of this glycolipid species. Furthermore, O antigens are polymerized structures with high molecular weights, making it increasingly difficult for analysis by mass spectrometry. We therefore chose a strain background containing a mutation in the O antigen chain length regulator (*wzz*) gene involved in efficient polymerization of O antigen subunits. This resulted in the production of mainly single repeat units and short polymerized O antigens, hence simplifying MS analysis. As mentioned previously, several other polysaccharide structures are also assembled on Und-PP, like peptidoglycan precursors, capsular polysaccharides and the enterobacterial common antigen (ECA), which might complicate the identification and characterization of O glycan species. We therefore used *E. coli* strain SCM6 for O antigen expression, which contains deletions in all major polysaccharide gene clusters.

With this modified method the O121 *wbqG* mutant O polysaccharides was analyzed. This study confirms the published struc-

ture by King *et al.* [159]. Furthermore, we could show that the recombinant expressed *wbqG* mutant O antigen structure contained O-acetylated N-acetylgalactosaminuronic acid, most likely modified at C-3. Therefore this mutant O polysaccharide contains structural motifs also present in the Vi. O-acetyl groups of the Vi polysaccharide form an immunodominant epitope and immunogenicity of Vi is closely related to the degree of O-acetylation [35,36].

For the first time it is reported that the *wbqG* mutant O polysaccharide is cross-reactive with antibodies raised against the Vi antigen. The strategy of using cross-reactive polysaccharide structures as vaccine components has been evaluated in several published studies. For example the cell wall polysaccharide (CWP) of the nonpathogenic bacterium *Bacillus pumilus* strain Sh18 was shown to be cross-reactive with the capsular polysaccharide (CPS) of *Haemophilus influenzae* type b. Conjugates containing the Sh18 CWP induced antibodies that reacted in an enzyme-linked immunosorbent assay (ELISA) with the CPS of *H. influenzae* type b strains [162]. Another study reported that *Shewanella* spp. CPSs share structural features with glycoproteins found in the *Bacillus anthracis* spore. A conjugate containing the *Shewanella* CPS induced antibodies that bound to *B. anthracis* spores and it will be further evaluated as a component of an anthrax vaccine [163].

Similarly, glycoconjugates composed of the *E. coli* O121 wild type or the *wbqG* mutant O polysaccharide and the *P. aeruginosa* exotoxin A (O121-EPA/ O121*wbqG*-EPA) were prepared in this study. EPA has already been successfully used as immunogenic carrier in a typhoid conjugate vaccine [41]. Both groups of mice immunized with glycoconjugates developed glycan specific antibody responses. 6 of 7 mice immunized with the O121*wbqG*-EPA conjugate showed a significant rise in serum immunoglobulin (Ig) anti-O121 LPS titer, indicating that other antigenic determinants than the uronamide groups are important for inducing an anti-O121 LPS specific immune response. Interestingly, antibodies of one animal immunized with the O121*wbqG*-EPA conjugate were not reactive with the *E. coli* O121 LPS but rather with the Vi poly-

saccharide. This implies that this animal developed an antibody response against the epitope constituted by residues b and c' (Figure 1), which resembles the Vi structure. However, the other animals of this group raised antibodies against an O121-LPS specific epitope, most likely residue d, containing a prominent surface exposed side group. Further optimizations of the O121 glycan structure might improve a Vi specific immune response upon immunization and experiments are planned to investigate if immunization with the O121*wbqG*-EPA conjugate primes a Vi-specific immune response that could be boosted with purified Vi polysaccharide.

Experimental Procedures

Bacterial strains, plasmids, and culture conditions

All bacterial strains and plasmids used in this study are listed in **Table I**. Construction of the plasmids is described below. *E. coli* strains were grown in LB medium (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter) or LB agar (LB medium with the addition of 15 g agar per liter) at 37 °C. *S.* Typhi BRD948 was grown in LB medium supplemented with 1 % v/v Aro-mix (40 mg L-phenylalanine, 40 mg L-tryptophan, 10 mg 4-aminobenzoic acid, and 10 mg 2,3-dihydroxybenzoic acid in 10 ml of ddH_2O) and 1 % v/v Tyr-mix (40 mg L-tyrosine disodium salt in 10 ml ddH_2O) at 37 °C. If appropriate, the media contained tetracycline (20 μg ml^{-1}), spectinomycin (80 μg ml^{-1}), or ampicilin (100 μg ml^{-1}).

Table I
Strains and plasmids used in this study.

Strain	Genotype or relevant description	Reference
S. Typhi BRD948	*S.* Typhi Ty2 Δ*aroC* Δ*aroD*	[60]
E. coli DH5α	K-12 φ80d*lacZ*Δ*M15 endA1 recA1 hsdR17*(rK–mK) *supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* F^-	Clonetech
E. coli O121	*Escherichia coli* serotype O121	CCUG 11422*
SCM6	SΦ874, Δ*wec* Δ*waaL*	C. Marolda and M. Valvano, unpublished
W3110	*rph-1 IN*(*rrnD-rrnE*)*1* λ^-	CGSC 4474**
Clm24	W3110, Δ*waaL*	[102]

Plasmid	Genotype or relevant description	Reference
pLAFR1	low copy-number broad host-range cosmid cloning vector; Tet^R	[140]
pEXT21	*tac* promoter expression vector; Sp^R	[172]
pGVXN157	pLAFR1 derivative with multiple cloning site inserted in *EcoR*I site; Tet^R	This study
pGVXN331	pGVXN157 derivative carrying O121 O antigen gene cluster of *E. coli* CCUG11422 on an *Asc*I/*Spe*I fragment; Tet^R	This study
pGVXN333	pGVXN331 derivative containing inactivated *wbqG*	This study
pGVXN121	pEXT21 derivative carrying *wecA*, IPTG inducible, Sp^R	This study
pGVXN150	Soluble periplasmic His_6-tagged toxoid variant (L552V, ΔE553) of *P. aeruginosa* exotoxin A (EPA) containing two engineered N-glycosylation sites cloned in pEC415, arabinose inducible, Amp^R	[105]
pGVXN114	HA-tagged *pglB* cloned in pEXT21, IPTG inducible, Sp^R	[105]

* Culture Collection University of Göteborg, Curator: Prof. E. R. B. Moore, Göteborg, Sweden

** The Coli Genetic Stock Center, Yale University, New Haven, CT, USA

DNA manipulations

Plasmid DNA was isolated using the NucleoSpin Plasmid or NucleoBond Xtra Maxi Plus kit (Macherey-Nagel). Total chromosomal DNA was isolated using NucleoSpin Tissue kit (Macherey-Nagel). Restriction enzymes (Fermentas), shrimp alkaline phosphatase (Fermentas), T4 DNA ligase (Fermentas), and Phusion High-Fidelity DNA polymerase (Finnzyme) were used according to the manufacturer's instructions. PCR and restriction fragments were purified for cloning using the NucleoSpin Extract II kit (Macherey-Nagel). All DNA sequencing was completed by Synergene Biotech GmbH (Switzerland) and synthetic oligonucleotides were ordered at Microsynth AG (Switzerland).

Plasmid constructions

pGVXN157 contains a synthetic oligonucleotide cassette formed from annealing of 5'- AATTGGCGCGCCCGGGACTAGTCTTGGG and 5'- AATTCCCAAGACTAGTCCCGGGCGCGCC ligated into the *Eco*RI-digested pLAFR1 [140], thereby introducing unique *Asc*I and *Spe*I single restriction sites. The *E. coli* O121 O antigen cluster was amplified from genomic DNA prepared from *E.coli* O121 (CCUG 11422) using the primers 5'- AAAGGCGCGCCGCGAAGGTAAAGTCAGCCG and 5'- AAAACTAGTCAGGAGTGAATTAAGTCATTG. The digested PCR fragment was ligated into the *Asc*I/*Spe*I digested pGVXN157 resulting in pGVXN331. pGVXN333 was constructed by inserting a synthetic oligonucleotide cassette formed from annealing of 5'-TGAATGAATGAACTAGTTCAATCACTCA and 5'-TGAGTGATTGAACTAGTTCATTCATTCA into the single restriction site *Pml*I, interrupting the open reading frame of *wbqG*.

LPS analysis

Cells of an overnight culture equivalent to an A_{600} of 1 were collected, resuspended in 100 µl of 1x sample buffer according to Laemmli [164] and boiled at 95 °C for 10 min. Proteinase K (Fermentas) was added to a final concentration of 200 µg ml^{-1} and the sample was incubated at 60 °C for 1 h. The LPS molecular species from the proteinase K-digested whole cell lysates were separated by SDS-PAGE using a 12 % BisTris NuPAGE gel from Invitrogen and MES running buffer according to manufacturer's instructions. LPS was visualized by staining with silver [165]. Immunological properties of O antigens were analyzed by Western blot using standard methods. The structure of the *E. coli* O121 O antigen is identical to the *Shigella dysenteriae* type 7 O antigen. Therefore, an anti-*S. dysenteriae* type 7 sera was purchased from Reagensia AB (Sweden) and used in a 1:100 dilution. Anti-Vi polyclonal antibody was purchased from Murex Biotech Ltd (England) and used in a 1:100 dilution.

Analysis of undecaprenyl pyrophosphate (Und-PP)-linked O specific polysaccharides

The O antigen glycans were analyzed in *E. coli* strain SCM6 (C. Marolda and M. Valvano, unpublished), which contains chromosomal deletions in several polysaccharide gene clusters. The O polysaccharide was expressed by transforming SCM6 cells with a plasmid encoding the O antigen cluster and the *wecA* expression plasmid pGVXN121. SCM6 transformed with empty plasmids was used as a negative control to identify O antigen specific signals. The strains were grown over night in a shake flask. Cells equivalent to an A_{600} of 400 were harvested, washed once with 0.9 % NaCl, and lyophilized. Lipids were extracted from the dried cells with 95 % methanol (MeOH) by repeated rounds of vortexing and incubation on ice for 10 min. The suspension was

converted into 85% MeOH by the addition of ddH_2O and further incubated for 10 min on ice while regularly vortexing. After centrifugation, the supernatant was collected and the extract was dried under N_2. The dried lipids were dissolved in 1:1 methanol/water (M/W) containing 10 mM tetrabutylammonium phosphate (TBAP) and subjected to a C_{18} SepPak cartridge (Waters Corp., Milford, MA). The cartridge was conditioned with 10 ml MeOH, followed by equilibration with 10 ml 10 mM TBAP in 1:1 M/W. After loading of the sample, the cartridge was washed with 10 ml 10 mM TBAP in 1:1 M/W and eluted with 5 ml MeOH followed by 5 ml 10:10:3 chloroform/methanol/water (C/M/W). The combined elution fractions were dried under N_2.

The lipid sample were hydrolyzed according to Glover *et al.* [166] by dissolving the dried samples in 2 ml 1 M trifluoroacetic acid (TFA) in 50% *n*-propanol and heating to 50°C for 15 min. The hydrolyzed sample was dried under N_2, dissolved in 4 ml 3:48:47 C/M/W and subjected to a C_{18} SepPak cartridge (Waters Corp., Milford, MA) to separate the lipids from the hydrolyzed glycans. The cartridge was conditioned with 10 ml MeOH, followed by equilibration with 10 ml 3:48:47 C/M/W. The sample was applied to the cartridge and the flow-through was collected. The cartridge was washed with 4 ml 3:48:47 C/M/W and the combined flow-through fractions were dried using a SpeedVac.

The dried samples were labeled with 2-aminobenzamide (2AB) according to Bigge *et al.* [167]. The glycan clean-up was performed using the paper disk method as described in Merry *et al.* [168]. The separation of 2AB-labeled glycans was performed by HPLC using a GlycoSep N normal phase column according to Royle *et al.* [169], but modified to a three solvent system. Solvent A: 10 mM ammonium formate pH 4.4 in 80% acetonitrile. Solvent B: 30 mM ammonium formate pH 4.4 in 40% acetonitrile. Solvent C: 0.5% formic acid. The column temperature was 30°C and 2AB-labeled glycans were detected by fluorescence (λex= 330 nm, λem= 420 nm). Gradient conditions: A linear gradient of 100% A to 100% B over 160 min at a flow rate of 0.4 ml min^{-1}, followed by 2 min 100% B to 100% C, returning to 100% A over

2 min and running for 15 min at 100 % A at a flow rate of 1 ml min^{-1}, then returning the flow rate to 0.4 ml min^{-1} for 5 min. samples were injected in ddH_2O.

To identify O antigen specific glycans, the 2AB glycan profile from cells carrying an empty plasmid control was subtracted from the trace obtained from cells harbouring an O antigen cluster. The O antigen specific peaks were collected and 2AB glycans were analyzed on a MALDI SYNAPT HDMS Q-TOF system (Waters Corp., Milford, MA). Samples were dissolved in 5:95 acetonitrile/water and spotted 1:1 with 20 mg ml^{-1} 2,5-dihydroxybenzoic acid (DHB) in 80:20 methanol/water. Calibration was done with PEG (Ready mixed solution, Waters Corp., Milford, MA), spotted 1:3 with 5 mg ml^{-1} α-cyano-4-hydroxycinnamic acid (CHCA, Sigma-Aldrich, Switzerland) in 60:40:0.1 acetonitrile/water/trifluoroacetic acid. The instrument was equipped with 200 Hz solid state UV laser. Mass spectra were recorded in positive ion mode. For MS-MS: laser energy was fixed at 240 at a firing rate of 200 Hz, collision gas was argon. A collision energy profile was used to ramp collision energy depending on the m/z. Combined, background subtracted, and smoothened (Savitzsky Golay) spectra were centered using MassLynx v4.0 software (Waters Corp., Milford, MA).

Production and purification of glycoconjugates

The production of glycoconjugates was achieved by expressing the oligosaccharyltransferase PglB, the engineered acceptor protein EPA (exotoxin A of *Pseudomonas aeruginosa*), and a gene cluster producing undecaprenyl-pyrophosphate (Und-PP)-linked glycans in *E. coli.* pGVXN114 (expressing PglB), pGVXN150 (expressing C-terminal His_6-tagged EPA) and pGVXN331 (O121 antigen cluster) or pGVXN333 (O121 *wbqG* mutant antigen) were co-transformed into *E. coli* strain Clm24 [102]. Cells were cultured in LB medium supplemented with antibiotics at 37 °C in the shaker incubator (180 rpm). Shake flask expression cultures were inocu-

lated from an uninduced overnight culture to an A_{600} of 0.05. Expression of PglB and the carrier protein EPA was induced at an A_{600} of 0.4–0.6 by IPTG (1 mM) and L-arabinose (0.02 % w/v). Four hours after the first induction a second pulse of L-arabinose (0.02 % w/v) was added. Cells were harvested after overnight incubation (total induction time of 19–22 h). Pellets were washed with 0.9 % NaCl and suspended in resuspension buffer (25 % sucrose, 10 mM EDTA, 200 mM Tris HCl pH 8.0) at a concentration equivalent to an A_{600} of 50. The cell suspension was incubated on a shaker for 20 min at 4 °C. After centrifugation the cell pellet was resuspended in the same volume of osmotic shock buffer (10 mM Tris HCl pH 8.0). The suspension was incubated on a shaker for 30 min at 4 °C and centrifuged at 10'000 g for 20 min to remove the spheroblasts. The supernatant containing periplasmic proteins was collected and the recombinant EPA containing a C-terminal hexahistidine tag was purified using a HisTrap crude FF 1 ml column (GE Healthcare, Switzerland). The extract was diluted with 5x HT binding buffer (2.5 M NaCl, 150 mM Tris HCl pH 8.0, 50 mM imidazole) to optimize the binding conditions and $MgCl_2$ was added to a final concentration of 50 mM. The extract was filtered and applied to the HisTrap crude FF column equilibrated with 1x HT binding buffer. After loading the column was washed with the same buffer containing 20 mM imidazole to remove unbound proteins. Proteins were eluted from the column with HT elution buffer (HT binding buffer containing 0.5 M imidazole).

Subsequently, the glycoprotein was separated from the unglycosylated EPA using a Resource Q 1 ml column (GE Healthcare, Switzerland). The HisTrap elution fractions containing EPA were pooled and diluted 10x with RQ binding buffer (20 mM L-histidine, pH 6.0). The diluted EPA sample was applied to the anion exchange column equilibrated with RQ binding buffer. The column was eluted with a linear gradient from 0 % to 32.5 % of RQ elution buffer (RQ binding buffer containing 1 M NaCl) in 25 column volumes and 0.5 ml fractions were collected using an Äkta FPLC (Amersham Biosciences). The fractions were analyzed by SDS-PAGE and proteins were stained with Coomassie blue.

Fractions containing glycoprotein were pooled and buffer was exchanged to PBS using an Amicon Ultra-4 centrifugal filter unit with a 30 kDa membrane (Millipore) by performing several concentration and dilution steps according to manufacturer's instructions. The concentration of the final purified protein sample was adjusted to 1 mg ml^{-1}.

Purification of *E. coli* O121 LPS

LPS of an *E. coli* O121 (CCGU 11422) culture was purified by phenol extraction as described elsewhere [170].

Purification of Vi polysaccharide and modification with tyramine

Vi polysaccharide was purified from *S.* Typhi BRD948 by a modified procedure as previously described [150]. Briefly, *S.* Typhi BRD948 was grown in LB medium supplemented with Aro- and Tyr-mix. After overnight incubation at 37 °C in the shaker incubator (180 rpm) the culture was heated to 60 °C for 1 h and centrifuged. Vi was precipitated from the supernatant with 0.1 % hexadecyltrimethylammonium bromide (CTAB, Sigma, H6269). 20 g l^{-1} celite 545 (Sigma, 20199-U) was added and the mixture was stirred for 1 h at room temperature (RT) in order to allow the formation of a polysaccharide-CTAB complex, which adsorbs onto the celite. The celite was poured into a reservoir of appropriate size (Extract-clean EV SPE Reservoir, Socochim S.A.) equipped with a frit (Socochim S.A.). The column was washed successively by gravity flow with 10 column volumes (CV) of 0.05 % CTAB, 10 CV of 20 % ethanol, 50 mM sodium phosphate buffer pH 6.0, and 14 CV of 45 % ethanol to eliminate adsorbed impurities. The Vi polysaccharide was finally eluted with 1.5 CV of 50 % ethanol, 0.4 M NaCl. Following elution, the polysaccharide was precipitated by

the addition of ethanol to a final concentration of 80 % and incubation for 20 min at RT. Finally, the precipitated polysaccharide was collected by centrifugation for 20 min at 15000 g, washed twice with 80 % ethanol, and lyophilized.

The protein and nucleic acid content of the purified Vi polysaccharide was determined by the bicinchoninic acid assay (BCA) and UV spectroscopy respectively. O-acetyl content was measured with acetylcholine as standard [136].

To increase the binding efficiency of the Vi to microtiter plates, the polysaccharide was tyraminated (Vi-Tyr). Tyramine hydrochloride (30 mg ml^{-1}, Sigma) was added to 10 mg of purified Vi. 100 µl of 0.5 M N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide HCl (Sigma) was added and the mixture was incubated at pH 4.9–5.1 for 3 h. The reaction mixture was dialyzed against ddH_2O.

Immunization studies

Groups of 7 CB6F1 female mice, 6–8 weeks old, were used in immunization experiments. Mice were immunized, subcutaneously, with 20 µg of glycoconjugate with Alum (Rehydragel LV- Aluminium Hydroxide, General Chemical) as adjuvant or 5 µg of Vi polysaccharide (Typhim Vi, Sanofi Pasteur MSD). Adjuvantation of the glycoconjugate was done just before immunization. Briefly, the purified glycoconjugates were diluted with PBS to a final concentration of 200 µg ml^{-1}, Alum (final amount of Al^{3+} corresponded to 0.6 mg ml^{-1}) was added, and the solution was gently mixed for 1 h at room temperature. Immunizations were performed on days 1, 22 and 57. Groups of mice normally received 100 µl doses of vaccines, corresponding to 20 µg of conjugate (protein). Blood samples were collected 10 days after the second and 10 days after the last immunization.

Enzyme-linked immunosorbent assay (ELISA) for murine antibodies

Flat bottom 96 well micro-titer plates (Nunc immuno PolySorb) were coated with 50 µl of 5 µg ml^{-1} *E. coli* O121 LPS or 5 µg ml^{-1} of tyraminated Vi (Vi-Tyr), diluted in PBS, at 4 °C overnight. The coating solution was poured away and the plate was submerged and vigorously agitated in 4000 ml of wash buffer (1x PBS with 0.05 % Triton X 100). This washing step was performed at least 4 times. Subsequently, the plate was dried by placing and spinning upside down in a micro plate rotor. This washing procedure was always applied in further washing steps. Each well was completely filled with 300 µl of blocking buffer (1x PBS with 2.5 % BSA (globulin free BSA, Sigma, A7030)) and incubated 2 h at room temperature (RT) on a plate shaker. After washing and drying the plate, dilutions of mouse serum in dilution buffer (1x PBS with 0.5 % BSA) were added to the plate (100 µl) and incubated 1 h at RT on a plate shaker. To detect total immunoglobulin (Ig), 100 µl of horseradish peroxidase (HRP) labeled goat anti-mouse Ig (Sigma) diluted 1:2000 in dilution buffer was added to each well and the plate was incubated for 1 h at RT on a plate shaker. Following washing and drying the plate, the reaction was developed with 100 µl of Ultra TMB substrate (3,3',5,5'-tetramethybenzidine liquid substrate, Pierce) for 15 min and stopped with the addition of 100 µl of 2 M sulphuric acid. Optical density (OD) was measured at 450 nm.

To determine the endpoint titer a 95 % confidence level was defined according to [171]. As negative sample a pool of preimmune sera was used.

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Relaxed sugar substrate specificity of the bacterial oligosaccharyltransferase PglB: transfer of the *Salmonella* Typhimurium O-antigen to an acceptor protein

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Contributions:

Construction of strains
Optimization of PglB expression
LPS analysis
Production and purification of glycoconjugates

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Abstract

The substrate specificity of the bacterial oligosaccharyltransferase (OST) PglB from *Campylobacter jejuni* towards the glycan moiety of the LLO substrate was extensively studied, including the structural requirements of the reducing end saccharide, which is directly involved in recognition and catalysis. These studies were conducted *in vivo* by recombinantly expressing PglB, an acceptor protein and making use of diverse bacterial glycan structures that are assembled on the lipid carrier undecaprenyl pyrophosphate (Und-PP), therefore serving as potential donor substrates in the glycosylation reaction. As a result of those studies it has been postulated that Und-PP linked glycans containing an N-acetyl group at the C-2 of the reducing end saccharide solely serve as substrates for the bacterial OST. However, the recombinantly expressed N-linked protein glycosylation system reconstituted in *Escherichia coli* did not reach the efficiency of glycosylation observed in the natural host. Therefore, previous work describing the substrate specificities of PglB using this *in vivo* glycosylation system might be inaccurate. In this study, the *in vivo* efficiency of glycosylation was improved by optimizing the conditions for recombinant PglB expression. With this improved system we re-examined the PglB-mediated transfer of an Und-PP-linked O-antigen from *Salmonella enterica* serovar Typhimurium containing a reducing end galactose. We have shown that: (i) PglB mediates the transfer of the *S.* Typhimurium O-antigen and (ii) that both species of the *S.* Typhimurium Und-PP-linked O-antigen substrate, *i.e.* the long form consisting of 16–35 repeating units and the very long form (VL) assembled of >100 subunits, serve as substrates for the glycosylation reaction.

Introduction

The post-translational modification of asparagine side chains with oligosaccharides (N-linked glycosylation) is a vital process in eukaryotes and involved in a multitude of cellular functions [173-175]. N-linked glycosylation occurs at the luminal side of the endoplasmatic reticulum (ER) membrane and is catalyzed by the oligosaccharyltransferase (OST), a hetero-oligomeric membrane protein complex [176]. OST catalyzes the formation of an N-glycosidic linkage by transferring the C1 carbon of the reducing end of the saccharide moiety of a preassembled lipid-bound $Glc_3Man_9GlcNAc_2$ oligosaccharide from the polyisoprenyl carrier lipid dolichyl pyrophosphate to the amide nitrogen atom of the acceptor asparagine residue in the glycosylation consensus sequon Asn-X-Ser/Thr (where X is any amino acid except proline) of polypeptide chains. In yeast (*Sacchoromyces cerevisiae*) OST consists of nine subunits of which Stt3p is the catalytically active component [177,178].

In recent years it has been shown that N-linked glycosylation is not restricted to eukaryotes, but is found in all domains of life. A homologous process in bacteria was first described in the foodborne pathogen *Campylobacter jejuni* [100,101]. Examination of genome sequence data demonstrated that N-glycosylation systems are widely spread in the δ and ε subdivisions of proteobacteria [179]. Compared to the eukaryotic pathway, biosynthesis of glycoproteins in prokaryotes is less complex. The bacterial OST, PglB, consists of a single subunit, which is homologous to the eukaryotic STT3 subunits. The N-linked glycosylation machinery of *C. jejuni* is encoded in a single gene cluster termed *pgl* (protein glycosylation) [180], which can be functionally transferred into *Escherichia coli* for recombinant glycoprotein expression [101]. *Pgl*-encoded enzymes assemble a heptasaccharide **(Figure 1)** attached to the polyisoprenyl carrier lipid undecaprenyl pyrophosphate (Und-PP), on the cytosolic face of the inner membrane [166,181-183]. The lipid-linked oligosaccharide (LLO) diffuses transversely to the periplasmic side by the ATP-binding cassette (ABC) transporter PglK [184], where the glycan is subsequently transferred to select-

***Campylobacter jejuni* N-glycan:**

α-D-GalNAc-(1→4)-α-D-GalNAc-(1→4)-α-D-GalNAc-(1→4)-α-D-GalNAc-(1→3)-β-Bac-(1→
3
↑
β-D-Glc(1

***Salmonella enterica* serovar Typhimurium O5:**

→2)-α-D-Man-(1→4)-α-L-Rha-(1→3)-α-D-Gal-(1→
3
↑
α-Abe(1
|2
OAc

***Shigella dysenteriae* O1:**

→3)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-α-D-Gal-(1→3)-α-D-GlcNAc-(1→

***Shigella flexneri* 2a:**

→2)-α-L-Rha-(1→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-β-D-GlcNAc-(1→
4
↑
α-D-Glc(1

Figure 1: Structure of the *Campylobacter jejuni* N-glycan and the repeating units of the *Salmonella* Typhimurium, *Shigella dysenteriae* O1, and *Shigella flexneri* 2a O-antigen.
Acetylation of the *Salmonella* Typhimurium O-antigen on the abequose moiety determines the O5 epitope. Bac: bacillosamine (2,4-diacetamido-2,4,6-trideoxy-D-glucose); Abe: abequose (3,6-dideoxy-D-galactose).

ed asparagine residues of periplasmic acceptor proteins by PglB. The optimal bacterial consensus sequence for N-glycosylation is Asp/Glu-Y-Asn-X-Ser/Thr (where Y and X are unspecified amino acid residues excluding proline) [104]. However, the presence of the sequon within a polypeptide chain is not sufficient for glycosylation. Acceptor sites that are located in rigid regions of the pro-

tein are suboptimal substrates for the bacterial OST. Grafting the sequon into flexible loops of the folded protein greatly enhances glycosylation efficiencies [185] and enables the production of recombinant glycoproteins with specified engineered glycosites. The recently published X-ray structure of the PglB orthologue from *Campylobacter lari* in complex with an acceptor peptide provided insights into glycosylation sequon recognition and amide nitrogen activation [186].

Besides recognition of the acceptor sequon, the OST has to bind the LLO substrate to complete catalysis. The structural requirements of the polyisoprenyl glycosyl carrier lipid and the oligosaccharide moiety were extensively studied in diverse *in vitro* and *in vivo* assay systems. Kinetic studies with a truncated *C. jejuni* N-glycan linked to polyisoprenyl carrier lipids that differed in polyisoprene chain length, degree of saturation, and double bond stereochemistry revealed that PglB exhibits distinct preferences, whereby *cis*-double bond geometry and α-unsaturation are important features of the polyisoprenyl carrier [187].

The vast variety of oligosaccharide structures that are assembled on Und-PP was used to study the structural requirements of the glycan moiety. It has been recognized that some of these polysaccharide biosynthetic pathways are highly homologous to the generation of the LLO substrate for bacterial N-glycosylation. The polymerase-dependent pathway for synthesis of O-antigens involves the assembly of oligosaccharides on Und-PP at the cytosolic side of the inner membrane. These LLOs are flipped to the periplasmic side by a flippase, Wzx, where the subunits are polymerized by Wzy polymerase. After polymerization the O-antigen polysaccharide is transferred to the lipid A-core by the ligase WaaL. It has been shown that PglB expressed in a *waaL* deficient mutant strain can accept a variety of different Und-PP linked O polysaccharides [102,103]. All of the PglB substrates identified so far contain either 2,4-diacetamido-2,4,6-trideoxyglucose (bacillosamine, Bac), D-GlcNAc, D-GalNAc or D-FucNAc at the reducing end [103]. A commonality of these sugars is the acetamido group at the C-2 position, which is also found in the glycan

substrate of eukaryotic OST complexes. Furthermore, the PglB-mediated transfer of Und-PP linked polysaccharides containing a galactose at the reducing end, like the O-antigen of *Salmonella enterica* subspecies I serovar Typhimurium (*S.* Typhimurium, Figure 1) was not observed [103]. Therefore, it was proposed that the acetamido group at the C-2 position plays a crucial role during catalysis either by interacting with the OST catalytic site through critical H-bonding or by stabilizing the oxonium intermediate after release of the undecaprenyl pyrophosphate leaving group [103]. Modeling of the LLO substrate into the PglB structure such that the 2,4-diacetamido-2,4,6-trideoxyglucose (Bac) moiety is properly aligned for a nucleophilic attack by the activated amide nitrogen, places the C-2 N-acetyl group of the first saccharide in the vicinity of a conserved tyrosine residue [186]. Therefore, additional H-bonding would favor a C-2 N-acetylated glycan substrate.

In this study the efficiency of glycosylation was improved by optimizing the recombinant expression of the bacterial OST. In this optimized system the substrate specificity of PglB was re-examined by transferring lipid-linked polysaccharides containing a hexose at the reducing end, *i.e.* the O-antigen of *S.* Typhimurium.

Results

Improving the efficiency of N-glycosylation in *E. coli* by optimizing recombinant expression of PglB.

Wacker *et al.* [101] complemented a *C. jejuni pglB* mutant strain by introducing the 16 kb protein glycosylation locus (*pgl*) on a plasmid. Analysis of the membrane protein extract revealed that the mutant phenotype was only partially complemented. The membrane bound periplasmic protein AcrA, containing two glycosites, is 100% glycosylated in the wild type *C. jejuni* strain, whereas in the complemented *pglB* mutant strain unglycosylated and monoglycosylated forms of AcrA were detected. Feldman *et al.* [102]

reconstituted the *C. jejuni* N-glycosylation machinery in *E. coli* by coexpressing the *pgl* locus and a soluble, periplasmic form of AcrA. However, the degree of protein glycosylation in the *E. coli* system did not reach wild type levels; periplamic protein extracts showed di-, mono-, and unglycosylated forms of AcrA. Furthermore, Feldman *et al.* [102] constructed a plasmid containing an HA-tagged PglB under the control of an arabinose inducible promoter in pMLBAD (pMAF10). The activity of the arabinose inducible *pglB* construct was tested by coexpressing the *pgl* gene cluster expressing the non-functional W458A, D459A PglB mutant (*pgl*$_{mut}$) and *acrA*. Also in this three-plasmid system wild type efficiency of glycosylation was not achieved.

We speculated that the availability of high levels of active PglB enzyme within the cell is crucial for efficient glycan transfer to acceptor proteins. For PglB expression optimization, the HA-tagged version from pMAF10 was subcloned into a variety of expression plasmids with different inducible promoters and copy numbers. Pre-experiments showed that subcloning of the OST into the low-copy plasmid pEXT21, thereby producing pGVXN114, was superior to all other constructs in terms of expression levels (results not shown). PglB expression in pGVXN114 is under control of the IPTG inducible *tac* promoter. Glycosylation efficiency of this expression system was compared to the pMAF10 construct by coexpression with the *pgl*$_{mut}$ gene cluster and the soluble form of AcrA in *E. coli* **(Figure 2A)**. Periplasmic proteins were extracted and AcrA was analyzed by SDS-PAGE and visualized by immunodetection. Mainly di-glycosylated AcrA was detected in presence of pGVXN114 whereas expression of the arabinose inducible PglB (pMAF10) resulted in incomplete modification of the periplasmic protein with the *C. jejuni* N-glycan. Furthermore, glycosylation efficiency was tested using a different Und-PP linked glycan structure, the *Shigella dysenteriae* O1 (O1$_{S.d.}$) O-antigen **(Figure 2B)**. The O1$_{S.d.}$ O-antigen contains a D-GlcNAc at the reducing end instead of the bacillosamine found in the *C. jejuni* N-glycan **(Figure 1)**. After induction of PglB, the C-terminally His6-tagged AcrA was purified from periplasmic extracts by affinity chroma-

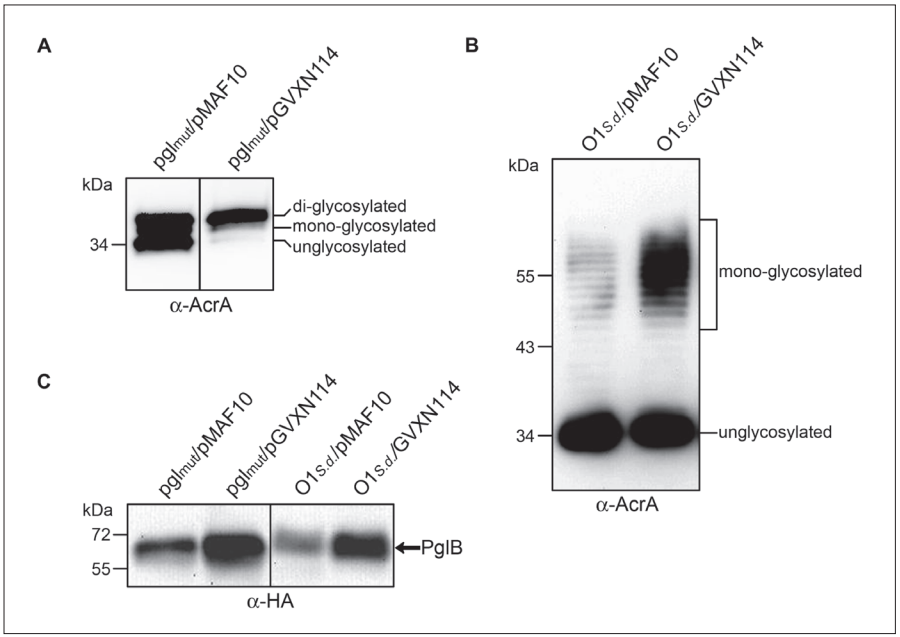

Figure 2: Improvement of glycosylation efficiency by optimizing the recombinant PglB expression in *E. coli* cells.

A Periplasmic proteins were extracted from *E. coli* CLM24 co-expressing *acrA*, the mutant *pgl* cluster of *C. jejuni* that encodes an inactive PglB (pgl_{mut}), and the HA-tagged wild-type PglB either encoded in pMLBAD (pMAF10) or pEXT21 (pGVXN114). Samples were analyzed by SDS-PAGE, transferred to nitrocellulose membranes and detected with antibodies directed against AcrA. Only partially modified AcrA was detected in cells containing pMAF10. Besides the di-glycosylated form also mono- and unglycosylated AcrA were detected. Cells harbouring pGVXN114 efficiently glycosylated AcrA and mainly the di-glycosylated form was observed.

B AcrA was purified from *E. coli* CLM24 transformed with either pMAF10 or pGVXN114 and expressing the *S. dysenteriae* O1 ($O1_{s.d.}$) O-antigen. Samples were analyzed by SDS-PAGE, followed by transfer to a nitrocellulose membrane and immunodetection using an anti-AcrA antibody.

C Whole-cell extracts of the expression cultures described in (A) and (B) were analyzed by SDS-PAGE and the HA-tagged PglB was detected by Western blot using an anti-HA antibody. The expression level of PglB encoded in the pGVXN114 was higher compared to the pMAF10 construct.

tography. Purified AcrA was analyzed by SDS-PAGE and after transferring to a nitrocellulose membrane visualized with an anti-AcrA antibody. A band of the same mass as that of unglycosylated AcrA (34 kDa) and a ladder of bands of higher molecular weight clustered around 55 kDa were stained by immunodetection. These larger polypeptides, but not the unmodified form of AcrA, were also detected by the anti- O1*S.d.* antiserum, indicating the covalent modification with the O1*S.d.* O-antigen (results not shown).

These results illustrated, that the efficiency of glycosylation was dramatically improved in both cases by using the IPTG inducible PglB expression system. Expression of the HA-tagged PglB was analyzed in all cultures by Western blot using an anti-HA antibody **(Figure 2C)**. In both cases, the expression level of PglB was significantly higher in cells carrying the pGVXN114 plasmid. However, we observed that cells carrying the IPTG inducible PglB construct displayed a reduced growth rate after induction compared to cells harboring the arabinose inducible plasmid pMAF10.

The *S.* Typhimurium O-antigen is a substrate for PglB.

Wacker *et al.* [103] reported that the *S.* Typhimurium O-antigen **(Figure 1)** and the *E. coli* K30 capsular polysaccharide were not transferred to AcrA. Both glycan structures contain a galactose at the reducing end. The optimized, recombinant PglB expression system was used to re-examine the substrate specificity of the bacterial OST. *S.* Typhimurium strain SL3749 was used to test the transfer of its O-antigen by coexpressing an acceptor protein and PglB. Strain SL3749 contains a non-functional WaaL O-antigen ligase thereby accumulating Und-PP linked glycan substrate as transfer to lipid A-core is inhibited.

First, the O-antigen structure expressed by *S.* Typhimurium SL3749 was analyzed. Western blot analysis of a proteinase K digested whole cell sample revealed that an anti-*S.* Typhimurium O5 (O5*S.T.*) sera reacted with the glycan structure, indicating the

expression of an O-acetylated variant of the O-antigen **(Figure 3A)**. The numbers of repeating units in the O-antigen polymer are clustered around a modal value determined by the chain length regulator Wzz. Each *wzz* specifies the synthesis of an O-antigen of a characteristic length. The *S.* Typhimurium chromosome encodes two *wzz* genes, giving rise to a bimodal chain length distribution of polymerized O-antigen: The long (L) form containing 16–35 repeating units and the very long (VL) form consisting of >100 subunits [188]. No O-antigen was detected in StGVXN1794 containing a *wbaP* knockout. WbaP is the enzyme catalyzing the reversible transfer of Gal-1-P to undecaprenyl phosphate, thereby initiating O-antigen assembly [189]. In order to confirm the structure of the expressed O-antigen repeating unit, purified, 2AB-labeled O-antigen glycans were analyzed using a high-performance liquid chromatography (HPLC) and mass spectrometry (MS) based method. Und-PP-linked oligosaccharides were extracted from StGVXN2556 containing a knockout of the O-antigen polymerase *wzy*. Deletion of *wzy* results in the accumulation of single O-antigen repeats, thereby facilitating MS analysis. As a control Und-PP-linked oligosaccharides of the *wbaP* knockout StGVXN1794 were extracted. The lipid-linked oligosaccharides were purified using a C_{18} SepPak column and treatment with mild acid specifically released Und-PP linked glycans. After an additional purification step using a C_{18} SepPak column, the glycans were labeled with 2-aminobenzamide (2AB) and subsequently separated by normal phase HPLC using a GlycoSep N column. **Figure 3B** shows the chromatogram of the area where subunits of O-antigens are expected to elute. Specific peaks were collected, and the 2AB-labeled glycans were analyzed by mass spectrometry **(Figure 3C and 3D)**. The glycan structures identified by mass spectrometry are indicated in **Figure 3B**.

The chromatogram of the 2AB-labeled glycans prepared from StGVXN2556, displayed a specific peak eluting at 24.1 min. In this peak fraction a molecule with a mass-to-charge ratio (m/z) of 781.30 was identified. The fraction with the retention time of 36.2 min contained a species with m/z of 739.30. The latter m/z

corresponded to the single charged, 2AB-labeled *S.* Typhimurium subunit. The difference between these two masses corresponded 42 Da, which is the mass difference between an O-acetyl and a hydroxyl group. These two species were subjected to collisionally induced dissociation (CID) MS-MS. The corresponding series of single charged fragment ions was consistent with glycosidic cleavage products from the 2AB-labeled *S.* Typhimurium subunit **(Figure 3C and 3D)**. Furthermore, the series of single charged fragment ions beginning at m/z 781.3 and ending at m/z 301.13 seen in **Figure 3C** indicated the presence of an O-acetylated dideoxyhexose. The m/z 301.13 corresponded to a 2AB-labeled hexose, hence confirming its presence at the reducing end.

Next, we co-expressed PglB (encoded in pGVXN114) and the engineered, His_6-tagged acceptor protein EPA (toxoid variant of the *Pseudomonas aeruginosa* exotoxin A) in *S.* Typhimurium SL3749. As a negative control, we used cells harboring a pEXT21 plasmid encoding the HA-tagged non-functional mutant version of PglB (PglBmut). After induction of PglB and the acceptor protein, EPA was purified from periplasmic extracts using affinity chromatography. Purified EPA was analyzed by SDS-PAGE and visualized by immunodetection **(Figure 4A)**. A band corresponding to unglycosylated EPA (70 kDa) and a ladder of bands of higher molecular weight clustered between 100 and 170 kDa were detected. These bands reacted with anti-EPA antibodies, indicating the presence of modified forms of EPA. These larger polypeptides, but not the unmodified form of EPA, were also detected by the anti-O5*S.T.* antiserum. The high molecular weight bands were absent in the sample purified from cells expressing PglBmut, suggesting the modification of EPA with the *S.* Typhimurium O-antigen (O5*S.T.*-EPA).

For further analysis, the glycoprotein was enriched and separated from the unmodified EPA using anion exchange chromatography. The protein samples were analyzed by SDS-PAGE and visualized by Coomassie blue staining or immunodetection **(Figure 4B)**. Presence of a band that was recognized by anti-EPA, but not by anti-O5*S.T.* antibodies confirmed that unglycosylated EPA was not

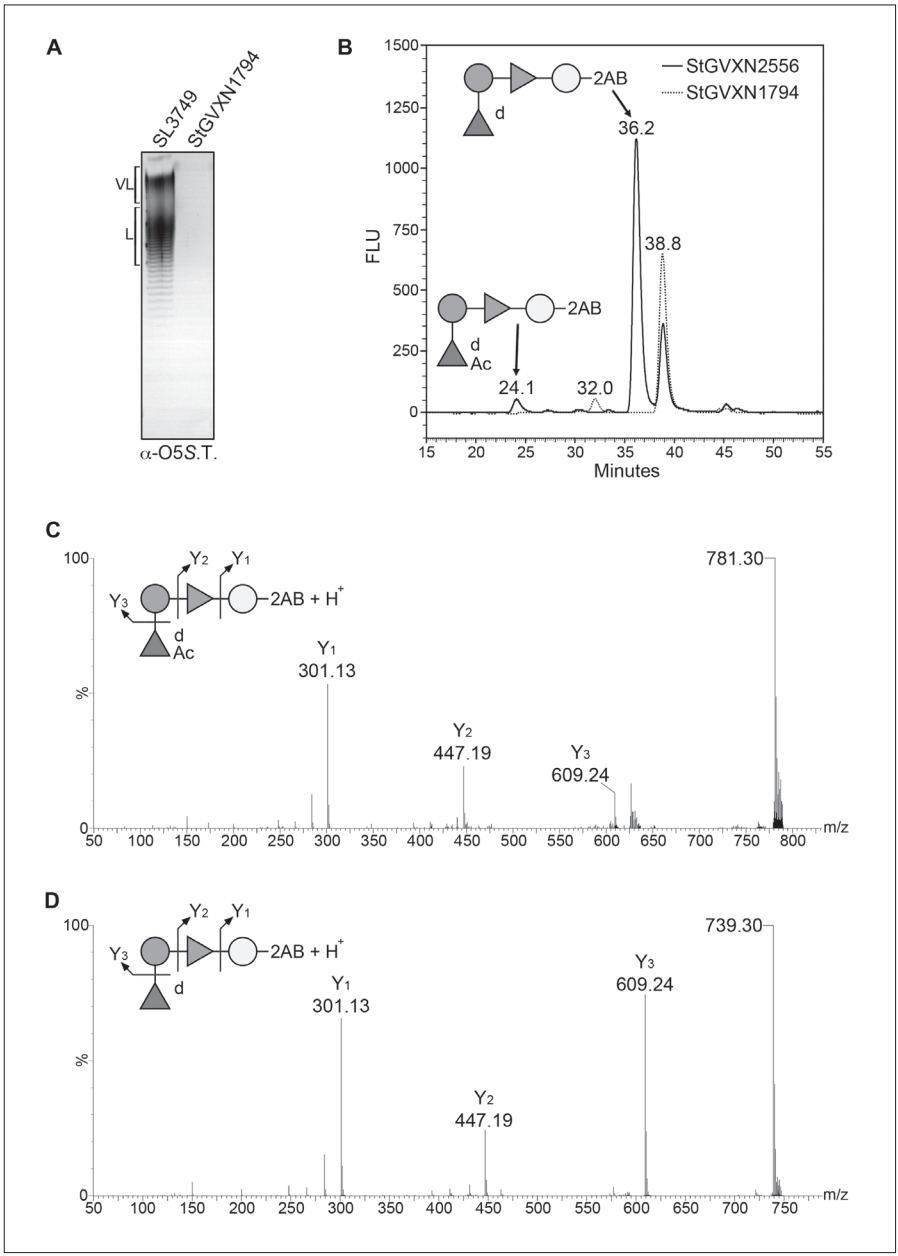
A
SL3749
StGVXN1794
VL
L
α-O5S.T.
B
StGVXN2556
StGVXN1794
2AB
d
36.2
38.8
2AB
d
Ac
24.1
32.0
FLU
Minutes
C
Y2
Y1
Y3
2AB + H+
d
Ac
781.30
Y1
301.13
Y2
447.19
Y3
609.24
%
m/z
D
Y2
Y1
Y3
2AB + H+
d
739.30
Y3
609.24
Y1
301.13
Y2
447.19
%
m/z

Figure 3: Analysis of the O-antigen expressed by *Salmonella* Typhimurium SL3749.

A Proteinase K digested whole-cell samples were analyzed by Western blot, using an anti-*S.* Typhimurium O5 (O5*S.T.*) antibody. A bimodal distribution of the O-antigen chain length is seen: the long form (L) containing 16–35 repeating units and the very long form (VL) consisting of >100 subunits. Deletion of *wbaP* in SL3749 (StGVXN1794) abolished O-antigen expression.
B Und-PP linked glycans were extracted from StGVXN2556 (SL3749, Δ*wzy*) and StGVXN1794 followed by 2AB labeling and separation by normal phase HPLC using a GlycoSep N column. The glycan structures detected in the O-antigen specific peaks are indicated. (white circle): hexose; (white right-pointing triangle): deoxyhexose; (white upwards-pointing triangle)[d]: dideoxyhexose; Ac: acetyl.
C CID MS-MS spectra of the main species detected in the peak eluting at 24.1 min.
D CID MS-MS spectra of the main species detected in the 36.2 min peak.

completely removed. However, most of the unmodified form was removed and based on the separation of the two species it was found that around 2% of the total EPA was glycosylated. In addition to a ladder of bands clustered between 100 and 170 kDa, glycosylated forms of EPA were detected with a molecular weight clearly higher than 170 kDa as seen by Commassie blue staining and detection with an anti-O5*S.T.* antibody.

Analysis of EPA glycosylated with the *S.* Typhimurium O-antigen.

The purified glycoprotein and the unmodified EPA seen in **Figure 4B** were subjected to high-mass MALDI ToF MS analysis **(Figure 5A)**. The molecular weight (MW) of the unmodified EPA (uEPA) was measured to be 68.78 kDa. The purified, glycosylated form of EPA (O5*S.T.*-EPA) had an average MW of 81.95 kDa. Furthermore, low amounts of unmodified EPA were also detected in the O5*S.T.*-EPA sample, but most of the unmodified form was

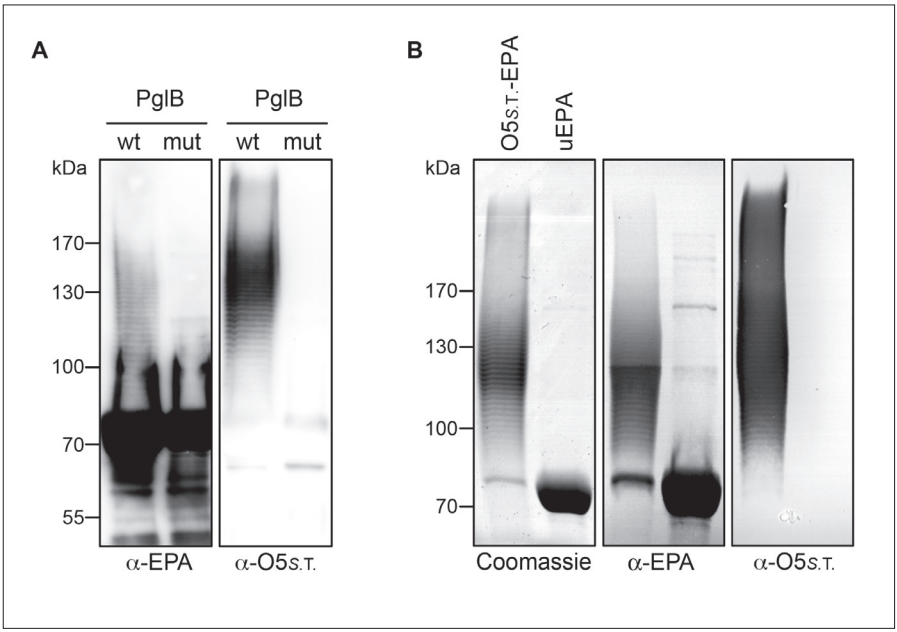

Figure 4: Glycosylation of EPA with the *S.* Typhimurium O5 polysaccharide.

A EPA was purified from *S.* Typhimurium SL3749 expressing PglB from pGVXN114 (wt) or PglB$_{mut}$ from pGVXN115 (mut). Purified proteins were analyzed by SDS-PAGE and after transfer to a nitrocellulose membrane visualized with antibodies directed against EPA or the *S.* Typhimurium O5 polysaccharide (O5$_{S.T.}$).

B EPA modified with the *S.* Typhimurium O5 polysaccharide (O5$_{S.T.}$-EPA) was further purified using anion exchange chromatography. O5$_{S.T.}$-EPA and the unmodified form of EPA (uEPA) were analyzed by SDS-PAGE followed by Coomassie blue staining or transfer to a nitrocellulose membrane and immunodetection with antibodies recognizing EPA, or the O5$_{S.T.}$ O-antigen.

successfully removed during anion exchange chromatography as previously seen in **Figure 4B**. No species of higher molecular weight were detectable in the O5*S.*T.-EPA sample. The difference in MW of the two protein samples therefore corresponds to the MW of the glycan moiety. By dividing the measured MW of the glycan by the calculated MW of an O-acetylated repeating unit of the *S.* Typhimurium O-antigen it was estimated, that the average length of the glycan attached to the protein consisted of around 22 subunits.

Next, the purified proteins were analyzed using High Performance Size Exclusion Chromatography (HPSEC). The chromatograms of uEPA and the glycosylated O5*S.*T.-EPA are shown in **Figure 5B**. In HPSEC the unmodified EPA elutes as a sharp peak at 15.41 min, whereas the main species of the glycosylated form elutes at 13.16 min as a broad peak. Furthermore, the O5*S.*T.-EPA displayed an additional peak eluting at 11.05 min indicating the presence of a larger species. Using a gel filtration standard, the MW of the differentially glycosylated species was determined. The MW of the unmodified EPA was calculated to be 70 kDa, whereas the O5*S.*T.-EPA main species had a MW of 202 kDa and the species eluting at 11.05 min had a MW of 548 kDa. The presence of two different species detected by HPSEC in the glycoprotein sample was apparently in conflict with the results obtained with high-mass MALDI ToF MS analysis where only a single species with an MW of 81.95 kDa was detected. Furthermore, the MW of the glycoprotein determined by gel filtration was significant higher as the one determined by MS which is mainly due to the rigid carbohydrate chain present in the glycoprotein, thereby drastically increasing its hydrodynamic volume.

We hypothesized that the two species detected by HPSEC in the O5*S.*T.-EPA sample corresponded to the carrier protein modified with a carbohydrate polymer of a bimodal chain length distribution. As seen in **Figure 3A**, *S.* Typhimurium expresses an O-antigen with these characteristics: the long form (L) which consists of 16–35 repeating units and the very long form (VL) assembled of >100 subunits.

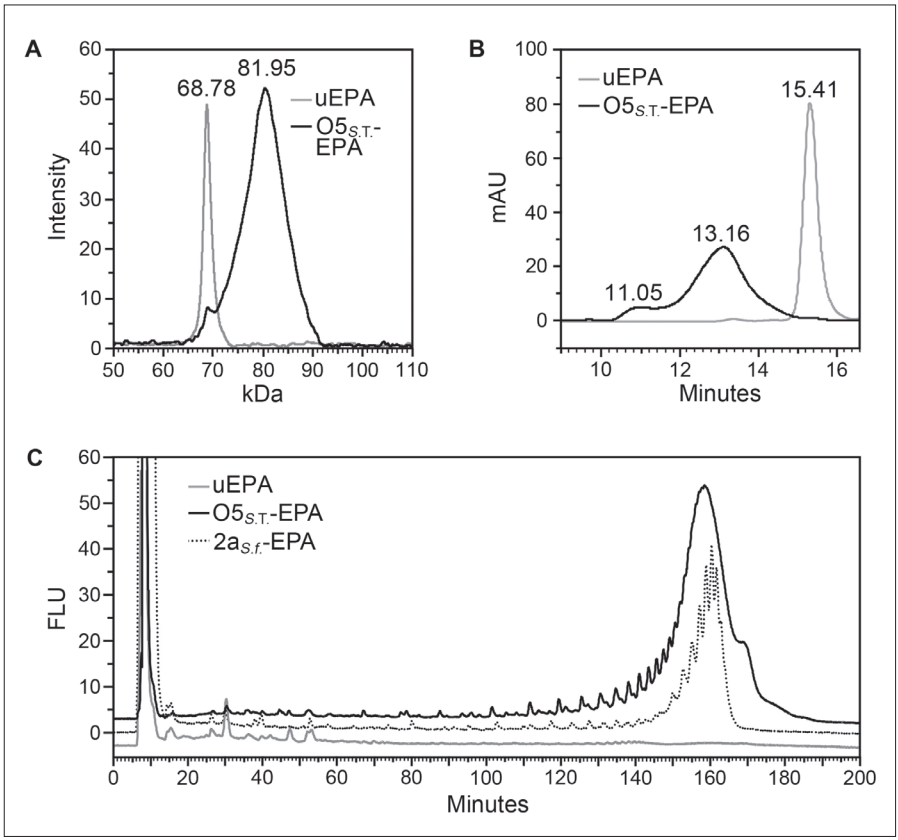

Figure 5: Analysis of the O5*S.T.*-EPA glycoprotein.

A High-mass MALDI ToF MS spectra of the O5*S.T.*-EPA and the unmodified EPA (uEPA).

B O5*S.T.*-EPA and uEPA were analyzed using High Performance Size Exclusion Chromatography (HPSEC). Two different species were detected in the O5*S.T.*-EPA sample.

C Analysis of the N-glycan, released from the glycoprotein by hydrazinolysis, followed by 2AB labeling and separation by normal phase HPLC using a GlycoSep N column. As a control, EPA modified with the *Shigella flexneri* 2a polysaccharide 2a*S.f.*-EPA was used. The released N-glycan of the 2a*S.f.*-EPA glycoprotein shows a modal, whereas the N-glycan of the O5*S.T.*-EPA conjugate displays a bimodal chain length distribution.

For characterization of the polysaccharide moiety in the glycoprotein the N-glycan was released by hydrazinolysis, followed by 2AB-labeling and separation by normal phase HPLC using a GlycoSep N column. In addition to the O5*S.*T.-EPA conjugate, a glycoprotein composed of EPA modified with the *Shigella flexneri* 2a O-antigen (2a*S.f.*-EPA) was analyzed. The structure of the *S. flexneri* 2a O-antigen is seen in **Figure 1**. The 2a*S.f.*-EPA glycoconjugate was produced in an *E. coli* W3110 strain background by coexpression of the *S. flexneri* 2a O-antigen cluster together with PglB and EPA. In contrast to *S.* Typhimurium, *E. coli* W3110 possesses a single Wzz protein, which confers to a modal chain length distribution of 11–17 repeating units. The unmodified EPA was used as a negative control. The chromatograms of the released, 2AB-labeled N-glycans are shown in **Figure 5C**. The *S. flexneri* 2a N-glycan showed a modal chain length distribution whereof glycans differing in a single repeating unit could still be separated. In contrast, the chromatogram of the *S.* Typhimurium N-glycan displayed an additional peak eluting at around 170 min, which is not seen in the *S. flexneri* 2a N-glycan chromatogram and which is indicative for a bimodal distribution of O-antigen chain length. Therefore, PglB is able to transfer both O-antigen species differing in length to the carrier protein EPA.

Discussion

The specificity of the bacterial oligosaccharyltransferase PglB from *C. jejuni* towards the glycan moiety of the LLO substrate was extensively studied. These studies have been conducted *in vivo* and are based on the following facts: (i) the bacterial N-linked glycosylation system can be reconstituted in *Escherichia coli* and (ii) bacteria assemble a vast variety of glycan structures on the lipid carrier undecaprenyl pyrophosphate, thus representing potential substrates of the bacterial OST. The substrate specificity of PglB was studied in an engineered system by co-expressing a polysaccharide biosynthetic gene cluster together with the bacterial OST and an acceptor protein. Especially the structural requirements of the reducing end saccharide, directly involved in recognition and catalysis, have been examined. As a result it has been postulated that Und-PP linked glycans containing an N-acetyl group at the C-2 of the reducing end saccharide solely serve as substrates for the bacterial OST. However, the N-linked protein glycosylation system reconstituted in *E. coli* did not reach the efficiency of glycosylation observed in the natural host. Therefore, previous work describing the substrate specificities of PglB *in vivo* might be inaccurate.

In this work the efficiency of glycosylation was improved prior to examinations concerning the substrate specificities of PglB. Increasing the concentration of substrates by over-expressing the periplasmic acceptor protein did not improve glycosylation (results not shown). Another strategy to boost the formation of glycoprotein involves the increase of LLO substrate. It might well be that LLOs are present at sub-saturating concentrations because the pool of the carrier lipid Und-PP is limited. Over-expression of undecaprenyl pyrophosphate synthase (UPPS), which catalyzes the consecutive condensation reactions of farnesyl pyrophosphate with eight isopentenyl pyrophosphates to generate Und-PP might lead to higher concentrations of this key lipid. In this work we found that the availability of high levels of active PglB is a critical factor for efficient catalysis. In addition to the low copy plasmid

pEXT21, other plasmid backbones were tested for PglB expression. We found that the IPTG inducible *tac* promoter was superior to the arabinose inducible promoter. Furthermore, gene dosage constitutes an additional important factor. For example, expression of PglB encoded in the high copy plasmid pEXT20 containing the same expression cassette as pEXT21, had a contrary effect on glycosylation and no glycoprotein was detectable.

This work shows that PglB is able to transfer an Und-PP linked glycan containing a reducing end galactose, but in case of the *S.* Typhimurium O-antigen, efficiency of glycosylation is lower compared to other polysaccharides that have been tested. Around 2% of the total extracted acceptor protein was glycosylated. In comparison, recombinant protein glycosylation with different O-antigen structures containing a HexNAc at the reducing end resulted in modification of up to 60–70% of the total extracted EPA. This study proves that the acetamido group at C-2 of the reducing end saccharide is not an essential requirement for PglB mediated transfer. Therefore, the proposed mechanism for catalysis, where the C-2 N-acetyl group is involved in stabilizing the oxonium intermediate after release of the pyrophosphate leaving group, is not plausible. However, due to the drastic difference in efficiency of glycosylation depending on the C-2 substitution of the reducing end sugar, it is likely that the acetamido group interacts with the OST enzyme through H-bonding. The substrate might thereby be bound and oriented properly in the active site for catalysis. The observed ability of PglB to transfer a glycan with a reducing end hexose opens up the possibility to engineer OST enzymes in order to improve the binding characteristics for such substrates.

Bacterial cell surface polysaccharides covalently linked to proteins have been developed as vaccines. These so-called conjugate vaccines are amongst the most effective and safe vaccines against bacterial diseases and have been used in humans for over 30 years. For example, a dramatic reduction in invasive pneumococcal disease in children and infants was observed after implementing vaccination using a conjugate vaccine [190]. The pneumococcal conjugate vaccine consists of the capsular polysaccharide of

Streptococcus pneumoniae (*S. pneumoniae*) covalently linked to the nontoxic diphtheria toxin CRM197. *Prevnar*, a pneumococcal vaccine developed by Wyeth (now Pfizer), is the world's best selling vaccine with sales of $3.7 billion in 2010 (Pfizer annual report 2010). However, the current vaccine covers only 13 of 90 different *S. pneumoniae* serotypes and an increase of infections caused by *S. pneumoniae* serotypes not covered by the 13-valent vaccine has been observed [191]. Novel vaccines that include new emerging strains are needed. The structure of most of the capsular polysaccharides of *S. pneumoniae* serotypes has been elucidated. Around 90% of the serotypes contain a hexose at the reducing end. The extended substrate specificity of PglB described in this report will facilitate the development of novel pneumococcal vaccines.

Experimental procedures

Bacterial strains, plasmids, and culture conditions

All bacterial strains and plasmids used in this study are listed in **Table 1**. Construction of the bacterial strains is described below. *E. coli* and *S.* Typhimurium strains were grown in LB medium (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter) or LB agar (LB medium with the addition of 15 g agar per liter) at 37°C. If appropriate, the media contained tetracycline (20 μg ml^{-1}), spectinomycin (80 μg ml^{-1}), trimethoprim (100 μg ml^{-1}), chloramphenicol (30 μg ml^{-1}) or ampicilin (100 μg ml^{-1}).

Table I
Strains and plasmids used in this study.

Strain	Genotype or relevant description	Reference
CLM24	*Escherichia coli* W3110; Δ*waaL*	[102]
SL3749	*Salmonella enterica* serovar Typhimurium LT2; *pyre(+), waaL446*	SGSC228*
StGVXN1794	SL3749; Δ*wbaP*	This study
StGVXN2556	SL3749; Δ*wzy*	This study

Plasmid	Genotype or relevant description	Reference
pMAF10	HA-tagged *pglB* cloned in pMLBAD, arabinose inducible, Tmp^R	[102]
pGVXN114	HA-tagged *pglB* cloned in pEXT21, IPTG inducible, Sp^R	[105]
pGVXN115	HA-tagged *pglB*$_{mut}$ encoding mutations W458A and D459A cloned in pEXT21, IPTG inducible, Sp^R	[105]
pACYC(*pgl*$_{mut}$)	encodes the *C. jejuni pgl* cluster cloned in pACYC184, encoding mutations W458A and D459A in PglB, Cm^R	[101]
pGVXN64	encodes the *S. dysenteriae* O1 *rfp* and *rfb* gene clusters cloned into pLAFR1, Tet^R	[105]
pWA2	Soluble periplasmic His_6-tagged *acrA* under control of *tet* promoter cloned in pBR322, Amp^R	[102]
pGVXN150	Soluble periplasmic His_6-tagged toxoid variant (L552V, ΔE553) of *P. aeruginosa* exotoxin A (EPA) containing two engineered N-glycosylation sites cloned in pEC415, arabinose inducible, Amp^R	[105]
pKD46	*γ*, *β* and *exo* from γ phage, *araC-ParaB*, Amp^R	[193]
pTOPO-DifCAT	Template plasmid for inactivation of chromosomal genes, contains cat gene flanked by *dif* sites, Kan^R, Clm^R	[192]

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DNA manipulations

Restriction enzymes (Fermentas) and Phusion High-Fidelity DNA polymerase (Finnzyme) were used according to the manufacturer's instructions. PCR products were purified using the NucleoSpin Extract II kit (Macherey-Nagel). All DNA sequencing was completed by Synergene Biotech GmbH (Switzerland) and synthetic oligonucleotides were ordered at Microsynth AG (Switzerland).

Construction of bacterial strains

Deletion of the chromosomal genes *wbaP* and *wzy* in *S.* Typhimurium LT2 strain SL3749 was done as described elsewhere [192,193]. Primers were designed containing 50 nucleotides of the 5' ends homologous to the chromosomal regions adjacent to the gene targeted for deletion. In addition the primers (5'- TAATATGCCTATTTTATTTACATTATGCACGGTCAGAGGGTGAGGATTAAATCTGCAGAATTCGCCCTTACC and 5'- TTTTACGCAGGCTAATTTATACAATTATTATTCAGTACTTCTCGGTAAGCAGTGTGCTGGAATTCGCCCTC used for *wbaP* deletion, 5'- TGCCTGATGGTAATATTTTTAATACTAAGCATTTTTTTCTAAAGGCTCTATATCTGCAGAATTCGCCCTTACC and 5'- ATTTTTACGCTTCAGAGCCAAATAAAACGGCGGCATTGCCGCCGTATAACAGTGTGCTGGAATTCGCCCTC used for *wzy* deletion) contained the underlined nucleotides that annealed to the template DNA from pTOPO-DifCAT, which carries a chloramphenicol resistance gene *cat*, flanked by *dif* (Xer recombinase recognition) sites. PCR products were purified, digested with *Dpn*I, repurified and suspended in 5 mM Tris pH 8.5 prior to further usage. SL3749 was first transformed with plasmid pKD46, which provides the λ Red gene functions for protection and integration of linear DNA placed under the control of the arabinose-inducible promoter P_{BAD}. Transformants carrying pKD46 were grown over-

night in 5 ml LB medium cultures with ampicillin at 30 °C in the shaker incubator. The overnight culture was diluted to an A_{600} of 0.05 in 50 ml LB medium containing ampicillin and L-arabinose (0.2 % w/v). At an A_{600} of 0.6 the cells were harvested and then made competent by washing once in 50 ml, twice in 25 ml and once in 1 ml ice cold ddH_2O. Finally, the cells were suspended in 200 µl of 10 % ice cold glycerol and the purified PCR products were introduced by electroporation. Chloramphenicol resistant colonies were screened by PCR with primers annealing to regions outside of the mutated gene and tested for ampicillin sensitivity to test for loss of the helper plasmid pKD46. The *cat* gene was excised from the chromosome by culturing the integrants in 50 ml LB medium without chloramphenicol. After periods of 24 hours, dilution series were plated on LB agar and the culture was inoculated into fresh LB medium. Single colonies were tested for chloramphenicol sensitivity and excision of the *cat* gene was verified by PCR with primers annealing to regions outside of the deleted gene. Furthermore, the PCR product of the region containing the deleted gene was sequenced.

Analysis of undecaprenyl pyrophosphate (Und-PP) linked O-antigen glycans

By Western blot: Cells of an over night culture equivalent to an A_{600} of 1 were collected, resuspended in 100 µl of 1x sample buffer according to Laemmli and boiled at 95 °C for 10 min. Proteinase K (Fermentas) was added to a final concentration of 200 µg ml^{-1} and the sample was incubated at 60 °C for 1 h. The LPS molecular species from the proteinase K digested whole cell lysates were separated by SDS-PAGE using a 12 % BisTris NuPAGE gel from Invitrogen and MES running buffer according to manufacturer's instructions. Immunological properties of O-antigens were analyzed by western blot using standard methods. Anti- *S.* Typhimurium O5 sera was purchased from the Statens Serum Institute

(SSI, Copenhagen, Denmark) and used in a 1:100 dilution.

By 2AB labeling: The O-antigen glycans were analyzed in StGVXN2556 containing a chromosomal deletion in the O-antigen polymerase *wzy* and a mutation in the O-antigen ligase *waaL*. The O-antigen glycan deficient strain StGVXN1794 (SL3749, Δ*wbaP*) was used as a negative control to identify O-antigen specific signals. Starting cultures were grown overnight in a shake flask. The overnight cultures were diluted to an A_{600} of 0.05 in 400 ml LB medium. At an A_{600} of 1.0, cells were harvested and washed once with 0.9% NaCl. The washed cell pellets were lyophilized. Lipids were extracted from the dried cells with 95% methanol (MeOH) by repeated rounds of vortexing and incubation on ice for 10 min. The suspension was converted into 85% MeOH by the addition of ddH_2O and further incubated for 10 min on ice while regularly vortexing. After centrifugation, the supernatant was collected and the extract was dried under N_2. The dried lipids were dissolved in 1:1 methanol/water (M/W) and subjected to a C_{18} SepPak cartridge (Waters). The cartridge was conditioned with 10 ml MeOH, followed by equilibration with 10 ml 10 mM TBAP in 1:1 M/W. After loading of the sample, the cartridge was washed with 10 ml 10 mM TBAP in 1:1 M/W and eluted with 5 ml MeOH followed by 5 ml 10:10:3 chloroform/methanol/water (C/M/W). The combined elutions were dried under N_2.

The lipid sample were hydrolyzed according to Glover *et al.* [166] by dissolving the dried samples in 2 ml 1 M trifluoroacetic acid (TFA) in 50% *n*-propanol and heating to 50 °C for 15 min. The hydrolyzed sample was dried under N_2, dissolved in 4 ml 3:48:47 C/M/W and subjected to a C_{18} SepPak cartridge (Waters) to separate the lipids from the hydrolyzed glycans. The cartridge was conditioned with 10 ml MeOH, followed by equilibration with 10 ml 3:48:47 C/M/W. The sample was applied to the cartridge and the flow through was collected. The cartridge was washed with 4 ml 3:48:47 C/M/W and the combined flow throughs were dried using a SpeedVac.

The dried samples were labeled with 2-aminobenzamide (2AB) according to Bigge *et al.* [167]. The glycan cleanup was performed

using the paper disk method as described in Merry *et al.* [168]. The separation of 2AB labeled glycans was performed by HPLC using a GlycoSep N normal phase column according to Royle *et al.* [169], but modified to a three solvent system. Solvent A: 10 mM ammonium formate pH 4.4 in 80% acetonitrile. Solvent B: 30 mM ammonium formate pH 4.4 in 40% acetonitrile. Solvent C: 0.5% formic acid. The column temperature was 30 °C and 2AB labeled glycans were detected by fluorescence (λex= 330 nm, λem= 420 nm). Gradient conditions: A linear gradient of 100% A to 100% B over 160 min at a flow rate of 0.4 ml min^{-1}, followed by 2 min 100% B to 100% C, returing to 100% A over 2 min and running for 15 min at 100% A at a flow rate of 1 ml min^{-1}, then returning the flow rate to 0.4 ml min^{-1} for 5 min. samples were injected in ddH_2O.

The O-antigen specific peaks were collected and 2AB glycans were analyzed on a MALDI SYNAPT HDMS Q-TOF system (Waters Corp., Milford, MA). Samples were dissolved in 5:95 acetonitrile/water and spotted 1:1 with 20 mg ml^{-1} 2,5-dihydroxybenzoic acid (DHB) in 80:20 methanol/water. Calibration was done with PEG (Ready mixed solution, Waters Corp., Milford, MA), spotted with 1:3 with 5 mg ml^{-1} α-cyano-4-hydroxycinnamic acid (CHCA, Sigma-Aldrich) in 60:40:0.1 acetonitrile/water/trifluoroacetic acid. The instrument was equipped with 200 Hz solid state UV laser. Mass spectra were recorded in positive ion mode. For MSMS: laser energy was fixed at 240 at a firing rate of 200Hz, collision gas was argon, a collision energy profile was used to ramp collision energy depending on the m/z. All spectra were combined, background subtracted, smoothed (Savitzsky Golay) and centred using MassLynx v4.0 software (Waters Corp., Milford, MA).

Production and purification of glycosylated AcrA

Production of glycoprotein was achieved by coexpressing *acrA* (pWA2), a gene cluster producing undecaprenyl-pyrophosphate (Und-PP) linked glycans (pACYC(pglmut) or pGVXN64), and the *Campylobacter jejeuni* oligosaccharyltransferase *pglB* (pMAF10 or pGVXN114) in *E. coli* CLM24 cells. Cells were cultured in LB medium supplemented with antibiotics at 37 °C in the shaker incubator (180 rpm). Shake flask expression cultures were inoculated from an uninduced overnight culture to an A_{600} of 0.05. Expression of PglB was induced at an A_{600} of 0.4–0.6 by the addition of either L-arabinose (0.2 % w/v in pMAF10 containing cells) or IPTG (1 mM in pGVXN114 containing cells). After induction at 37 °C for 4 h the cells were harvested by centrifugation and periplasmic proteins were extracted by osmotic shock extraction. Therefore, pellets were washed with 0.9 % NaCl and suspended in resuspension buffer (25 % sucrose, 10 mM EDTA, 200 mM Tris HCl pH 8.0) at a concentration equivalent to an A_{600} of 50. The cell suspension was incubated on a shaker for 20 min at 4 °C. After centrifugation the cell pellet was resuspended in the same volume of osmotic shock buffer (10 mM Tris HCl pH 8.0). The suspension was incubated on a shaker for 30 min at 4 °C and centrifuged at 10'000 g for 20 min to remove the spheroblasts. The supernatant containing the periplasmic proteins was collected and the hexahistidine-tagged AcrA was purified by affinity chromatography. The periplasmic protein extract was diluted with 5x HT binding buffer (2.5 M NaCl, 150 mM Tris HCl pH 8.0, 50 mM imidazole) to optimize the binding conditions and $MgCl_2$ was added to a final concentration of 50 mM. The extract was sterile filtered and applied to the HisTrap crude FF column equilibrated with 1x HT binding buffer and washed with the same buffer containing 20 mM imidazole to remove unbound proteins. Proteins were eluted from the column with HT elution buffer (HT binding buffer containing 0.5 M imidazole).

Production and purification of EPA glycosylated with the *S.* Typhimurium O-antigen

pGVXN114 (encoding PglB) and pGVXN150 (encoding hexahistidine-tagged EPA) were co-transformed into *S.* Typhimurium LT2 strain SL3749. Cells were cultured in LB medium supplemented with antibiotics at 37 °C in the shaker incubator (180 rpm). Shake flask expression cultures were inoculated from an uninduced overnight culture to an A_{600} of 0.05. The expression of *pglB* and *epa* was induced at an A_{600} of 0.4–0.6 by IPTG (1 mM) and L-arabinose (0.02 % w/v). Four hours after the first induction a second pulse of L-arabinose (0.02 % w/v) was added. Cells were harvested after overnight incubation (total induction time of 19-22 h). The periplasmic proteins were extracted by osmotic shock extraction and the hexahistidine-tagged EPA was purified by affinity chromatography as described above.

After purification of EPA, the glycoprotein was separated from the unglycosylated form using a Resource Q column (GE Healthcare). The HisTrap elution fractions containing EPA were pooled and diluted 10x with RQ binding buffer (20 mM Tris HCl pH 8.0). The diluted EPA sample was applied to the anion exchange column equilibrated with RQ binding buffer. The column was eluted with a linear gradient from 0 % to 32.5 % of RQ elution buffer (RQ binding buffer containing 1 M NaCl) in 25 column volumes and 0.5 ml fractions were collected using an Äkta FPLC (Amersham Biosciences). The fractions were analyzed by SDS-PAGE and proteins were stained with Coomassie blue. Fractions containing glycoprotein were pooled and buffer was exchanged to 1x phosphate buffered saline, pH 7.5 (PBS) using an Amicon Ultra-4 centrifugal filter unit with a 30 kDa membrane (Millipore) by performing several concentration and dilution steps according to manufacturer's instructions. The concentration of the final purified protein sample was adjusted to 1 mg ml^{-1} and was used for analytics.

Western blotting

Western blotting was performed using standard methods. Anti-AcrA antibodies are described in [8]. Antiserum against *S.* Typhimurium O5 was obtained from Statens Serum Institute (SSI, Sopenhagen, Denmark) and used in a 1:100 dilution. Antiserum against EPA was obtained from Sigma (Switzerland, P2318). Anti-HA-tag antibodies were obtained from Sigma (Switzerland, H6908).

High-mass MALDI ToF MS analysis

Mass spectrometry of intact proteins was performed by CovalX (Zurich, Switzerland). The high mass measurements were performed using a Reflex IV MALDI ToF mass spectrometer (Bruker, Bremen, Germany) equipped with CovalX's HM2 high-mass detector system.

Several dilutions of the protein were mixed 1:1 with a sinapinic acid matrix (10 mg ml^{-1}, in acetonitrile/ water 1:1, 0.1 % trifluoracetic acid). 1 µl of each sample was spotted on the MALDI plate (SCOUT 384, AnchorChip). After cristallization at room temperature the plate was introduced in the MALDI mass spectrometer. The high mass MALDI ToF MS analysis has been performed using the standard nitrogen laser and focusing on different ranges from 0 to 1200 kDa. Mass spectra were recorded in linear and positive mode. For the analysis the following parameters have been applied for the mass spectrometer: Ion source 1: 20 kV; Ion source 2: 17 kV; Lens: 12 kV; Pulse ion extraction: 400 ns. Calibration was done with clusters of insulin, BSA and IgG. For each sample, 3 spots were analyzed (200 laser shots per spot). The presented spectrum corresponds to the sum of 200 laser shots. MS data were analyzed using Complex Tracker 2.0 analysis software.

Molecular size determination of proteins by High Performance Size Exclusion Chromatography (HPSEC)

The molecular size of proteins was determined using a HPLC LaChrome Elite (Hitachi) equipped with a UV-VIS detector (L-2420). Samples were separated on a Supelco TSK-Gel G3000SWXL (Sigma/Supelco 808541) with a Supelco TSK-Gel SWXL (Sigma/Supelco 808543) guard column and detected by UV at 215 nm. The protein samples were diluted with 1x PBS, pH 7.5 to a concentration of 100 μg ml^{-1} and 50 μl were injected. The column oven temperature was adjusted to 25 °C, and the sample was run at a flow rate of 0.6 ml min^{-1}, isocratic in PBS, pH 7.5. To determine the void volume of the column the DNA molecular weight marker II (Roche, 102 3625 0001) was used and the retention time of the 23'130 bp fragment was measured. For determination of the inclusion volume the retention time of the sodium azide (Sigma, 71289) peak was measured. As a molecular size standard the MWGF1000 (29–669 kDa, Sigma, MWGF1000-1KT) diluted to 1.333 mg ml^{-1} in 1x PBS was used.

Release of N-linked glycans by hydrazinolysis

N-glycans were released using the Ludger Liberate Hydrazinolysis Glycan Release Kit (Ludger Ltd, LL-HYDRAZ-A2) according to manufacturer's instructions. The dried glycan samples were labeled with 2-aminobenzamide (2AB) according to Bigge *et al.* [167]. The glycan cleanup was performed using the paper disk method as described in Merry *et al.* [168]. The separation of 2AB labeled glycans was performed by HPLC using a GlycoSep N normal phase column as described above.

General Discussion

The production of novel glycoconjugates using the bacterial N-glycosylation system would enable the development of novel, cost efficient vaccines against diverse bacterial infections. In this thesis the feasibility of using this novel *in vivo* conjugation process for production of vaccines against *Salmonella* spp. infections was explored. As outlined in the introduction section, the antigenic cell surface polysaccharide structures of *Salmonella* are composed of the O and Vi antigens. Expression of O antigens is common to all *Salmonella* serovars, whereas expression of the Vi antigen, a capsular polysaccharide, is largely restricted to *S. enterica* serovar Typhi, the causative agent of typhoid fever. The O antigen structures of *S. enterica* serovars causing typhoidal and nontyphoidal salmonellosis in humans are closely related and share a common backbone. Even though these antigenic cell surface structures are validated targets for vaccine development, no commercial available conjugate vaccines exist to date. However, various conjugate vaccine candidates have been successfully tested in clinical trials and have been shown to be safe, immunogenic and effective [77-83,92,93,96,97].

For the development of conjugate vaccines using the bacterial N-glycosylation system, accessibility of these immunogenic cell surface polysaccharide structures to the bacterial oligosaccharyltransferase PglB, the key enzyme forming the N-glycosidic linkeage, is fundamental. For being a glycan substrate for PglB several requirements have to be fulfilled: (i) the glycan has to be assembled on the lipid carrier undecaprenyl pyrophosphate (Und-PP), (ii) during the biosynthetic pathway of the antigenic cell surface polysaccharide structure a Und-PP linked glycan precursor has to be localized in the periplasmic leaflet of the bacterial inner cell membrane and (iii) the dogma exists that the reducing end saccharide of the Und-PP-linked glycan structure must contain an acetamido group at the C-2 position, thought to be crucial for catalysis [103].

The first part of this thesis deals with the development of a conjugate vaccine against typhoid fever. Such a vaccine is composed of the Vi antigen bound to a carrier protein. The majority of publications that deal with the Vi capsule focus on regulation of expression during the different stages of infection and its contribution to the onset of typhoid fever. Little is known about the biosynthesis of the Vi capsule. Key aspects including initiation, elongation, export and cell surface retention of this important polysaccharide structure remain equivocal and its accessibility to the bacterial OST is unresolved. Therefore, a molecular characterization of the *viaB* locus encoding the biosynthetic machinery for Vi capsule formation has been done using *E. coli* as a surrogate system. The study provides a comprehensive phenotypic analysis of single gene transposon insertion mutants using diverse microscopic and biochemical techniques. The work shows that despite sharing the basic mechanism also found for *E. coli* group 2 capsule assembly, biosynthesis of the Vi capsule displays features uniquely found in this cell surface polysaccharide structure. The *viaB* locus encodes a gene, *vexE*, containing an acyltransferase domain. Inactivation of *vexE* resulted in a cell, which exports the polysaccharide to the outside but once translocated, the Vi is not retained at the cell surface and no capsule is formed. This phenotype has not yet been described for *E. coli* group 2 capsules nor do *E. coli* group 2 capsular gene clusters encode genes with homologous functions. However, *E. coli* group 2 capsular gene clusters encode genes that are believed to be involved in CMP-Kdo synthesis and the speculative addition of a diacylglycerophosphate-Kdo to the CPS. The presence of non-homologous genes encoded in the respective capsular gene clusters might reflect a different mechanism of polysaccharide lipidation, resulting in a different structure that links the glycan to the cell surface. Identification of this lipid anchor is important in elucidating the biosynthetic pathway of this class of polysaccharides. A possible strategy to identify such a lipid modification, which is most likely present at the reducing end of the glycan polymer, involves the enzymatic degradation of purified Vi. A source for such enzymes might be found in phages using the Vi as

receptor. Analysis of the enzymatically degraded capsular polysaccharide using mass spectrometry might result in the identification of the modified reducing end saccharide.

Another important aspect of Vi biosynthesis is the initiation mechanism, *i.e.* the starting structure that is elongated. It remains equivocal if expression of the Vi antigen is dependent on genes encoded outside of the *viaB* locus. This seems to be a common characteristic in bacterial glycan biosynthetic pathways as for example expression of the O antigen in *E. coli* strains is dependent on the initiating glycosyltransferase WecA, encoded in the enterobacterial common antigen (ECA) gene cluster. It might well be, that the biosynthetic machinery for Vi assembly is dependent on intermediates formed by other biosynthetic pathways. During this thesis, the *viaB* locus was expressed in several *E. coli* strains containing deletions in different cell surface glycan gene clusters, *e.g.* ECA locus, O antigen locus, colanic acid gene cluster. It was found that in all these mutant strains the Vi is expressed and that Vi expression is independent of all known initiating glycosyltrasferases resulting in Und-PP-linked sugars (results not shown).

Another approach to identify genes involved in Vi expression, which are encoded outside of the *viaB* locus, was based on a saturating whole genome mutagenesis screen to detect *S.* Typhi genes that when inactivated by transposon insertion resulted in escape from killing by the lytic Vi bacteriophage, which binds to the Vi for initiating infection. The location of transposon insertions that resulted in escape from killing was determined by transposon directed insertion site sequencing (TraDIS) (Pickard D, *et al.* manuscript in preparation). The Vi phage based screening of a *S.* Typhi library resulted in the identification of several genes involved in Vi biosynthesis and cell surface localization of the capsular polysaccharide, a number of genes encoding regulators, and genes of unknown function. However, genes involved in Vi biosynthesis and cell surface localization were identified exclusively within the *viaB* locus and no additional genes with glycan metabolic functions were identified outside the *viaB* locus. This implies that either all of the necessary genes are encoded within the *viaB* locus or that

essential or duplicated genes, which cannot be identified using this screening method, contribute to Vi capsule assembly. For instance it might be possible that the Vi biosynthetic machinery assembles the Vi polymer on the glycolipid intermediate undecaprenyl phosphate (Und-P)-glucose. Und-P-glucose is formed by GtrB, which is part of a prophage-encoded system involved in glucosylation of the O antigen and it is found in many other species like *E. coli* [112]. The gene product is proposed to catalyze the transfer of glucose from UDP-glucose to undecaprenyl phosphate (Und-P) at the cytosolic side of the inner membrane, resulting in the formation of Und-P-glucose [113,114]. The *S.* Typhi genome encodes two copies of *gtrB* and therefore it might not be identified as being involved in Vi biosynthesis by the whole genome mutagenesis approach. Interestingly, *oxyR*, which has been identified with the whole genome high-density mutagenesis screen, encodes a regulator not only involved in Vi locus down regulation, but also in the down regulation of the *gtr* loci (Pickard D, personal communication).

As outlined above, there are no indications that the Vi polymer is assembled on the lipid carrier Und-PP. Furthermore microscopic studies of cells containing mutations in different parts of the Vi translocation machinery did not show periplasmic accumulations of the polysaccharide. Because these points are not fulfilled for the Vi antigen, it is not thought to be a substrate for PglB. Therefore a different strategy was implemented for the development of a typhoid vaccine candidate using the bacterial N-glycosylation system by searching for Und-PP-linked glycan structures that contained Vi epitopes. Such a structure was found in the O antigen of *E. coli* O121 [158,159]. It could be shown that a mutant variant of the O121 O antigen is recognized by anti Vi antibodies and therefore is cross-reactive with Vi. The glycoconjugate induced good antibody titers against wild type *E. coli* O121 O antigen. However the mutant O antigen, which cross-reacts with Vi, was not able to induce a significant anti Vi response. These results indicate that antigenic cross-reactivity might not be sufficient for the induction of a specific and functional antibody response against a na-

tive virulence factor. The observation that the same animal, which did not induce anti *E. coli* O121 antibodies mounted an anti Vi response, might indicate that a very specific epitope (not cross-reacting with O121 O antigen) must be presented to the immune system to drive the response against Vi. In order to improve an anti Vi immune response, the cross-reactive structure should be further optimized. For example by additionally deleting the glycosyltransferase adding the Qui4NAcGly residue to the repeating unit of the O antigen (see Figure 1, page 91). This would result in a trisaccharide mimicking the Vi and containing fewer O121 specific epitopes. Furthermore this lipid-linked glycan intermediate might represent a starting structure for the processive glycosyltransferase encoded within the *viaB* locus, which might elongate the trisaccharide resulting in an Und-PP-linked Vi antigen. Once the engineered Vi antigen is flipped to the periplasm it would be accessible to the oligosaccharyltransferase.

In the second part of this thesis the *in vivo* production of glycoconjugates consisting of the *S. enterica* O antigen bound to a protein carrier was investigated. In contrast to the Vi antigen, the biosynthetic pathway of O antigen assembly has been elucidated [110]. This class of cell surface glycan structures is assembled on Und-PP and periplasmic intermediates exist. However, a commonality of the O antigens expressed by different important *S. enterica* serovars causing human illnesses is a galactose at the reducing end of the O antigen subunit. It has been reported that glycan structures containing a hexose at the reducing end are not transferred by the bacterial OST due to the lack of the acetamido group at C-2, which was thought to be essential for catalysis [103]. Nonetheless, it was shown in this work that the O antigen of *S.* Typhimurium was transferred to acceptor proteins upon optimization of recombinant PglB expression. Therefore, the acetamido group at C-2 of the reducing end saccharide is not an essential requirement for PglB mediated transfer. This important observation creates the opportunity to develop novel conjugate vaccines against several *S. enterica* serovars expressing closely related O specific polysaccharide structures, *e.g. S.* Paratyphi A. As outlined in

the introduction, the lack of effective vaccines against *S*. Paratyphi A is of great concern because this serotype appears to be responsible for a growing proportion of enteric fever. Implementation of vaccination against *S*. Typhi aggravated this trend and it raised concerns whether the introduction of a vaccine against *S*. Typhi, in the absence of a vaccine against *S*. Paratyphi A, has any impact at all on enteric fever. Conjugate vaccine candidates consisting of the *S*. Paratyphi A O antigen bound to tetanus toxoid were shown to be safe and immunogenic in humans [92,93]. Production of conjugate vaccines consisting of the O antigen bound to carrier proteins is a complex process due to the additional detoxification step of the LPS prior to chemical coupling. Additionally, the *S*. Paratyphi A O antigen contains immunodominant O-acetyls that might be lost during chemical conjugation. The *in vivo* conjugation technology could overcome these difficulties, making *S*. Paratyphi A the ideal candidate for vaccine development.

Even though the lack of a N-acetyl group at the reducing end of the glycan substrate does not make PglB mediated catalysis impossible, it has a dramatic effect on transfer efficiency. Therefore it is crucial to improve the transfer efficiency of this class of glycan structures first prior to vaccine development. This might be achieved by optimizing the fermentation conditions of the glycoconjugate production strain. Different parameters have to be screened that improve optimal expression of all components of the *in vivo* conjugation process. Additionally, due to the fact that the acetamido group at C-2 is not essential for the catalytic mechanism, PglB could be optimized for transferring this class of glycan structures by a directed evolution approach. Therefore a high throughput screening method has to be implemented. Improving the glycosylation efficiency would enable the further optimization of immunogenic glycoconjugates.

References

1. Kauffmann F (1941) Die Bakteriologie der *Salmonella* Gruppe. Copenhagen: Ejnar Munksgaards Forlag.
2. Grimont PAD, Weill F-X (2007) Antigenic Formulae of the *Salmonella* Serovars. WHO Collaborating Center for Reference and Research on *Salmonella* 9th edition.
3. Baumler AJ, Tsolis RM, Ficht TA, Adams LG (1998) Evolution of host adaptation in *Salmonella enterica*. Infect Immun 66: 4579–4587.
4. Groisman EA, Ochman H (1996) Pathogenicity islands: bacterial evolution in quantum leaps. Cell 87: 791–794.
5. Lostroh CP, Lee CA (2001) The *Salmonella* pathogenicity island-1 type III secretion system. Microbes Infect 3: 1281–1291.
6. Zhou D, Galan J (2001) *Salmonella* entry into host cells: the work in concert of type III secreted effector proteins. Microbes Infect 3: 1293–1298.
7. Muller AJ, Kaiser P, Dittmar KE, Weber TC, Haueter S, *et al.* (2012) *Salmonella* gut invasion involves TTSS-2-dependent epithelial traversal, basolateral exit, and uptake by epithelium-sampling lamina propria phagocytes. Cell Host Microbe 11: 19–32.
8. Miller SI, Hohmann EL, Pegues DA (1995) *Salmonella* (including *Salmonella typhi*). In: Madell GL, Bennett JE, Dolin R, editors. Principles and practice of infectious diseases. 4th ed. ed. New York: Churchill Livingstone. pp. 2013–2033.
9. Parkhill J, Dougan G, James KD, Thomson NR, Pickard D, *et al.* (2001) Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. Nature 413: 848–852.
10. Voetsch AC, Van Gilder TJ, Angulo FJ, Farley MM, Shallow S, *et al.* (2004) FoodNet estimate of the burden of illness caused by nontyphoidal *Salmonella* infections in the United States. Clin Infect Dis 38 Suppl 3: S127–134.

11. Gordon MA (2011) Invasive nontyphoidal *Salmonella* disease: epidemiology, pathogenesis and diagnosis. Curr Opin Infect Dis 24: 484–489.
12. Hornick RB, Greisman SE, Woodward TE, DuPont HL, Dawkins AT, *et al.* (1970) Typhoid fever: pathogenesis and immunologic control. 2. N Engl J Med 283: 739–746.
13. Giannella RA, Broitman SA, Zamcheck N (1972) Gastric acid barrier to ingested microorganisms in man: studies *in vivo* and *in vitro*. Gut 13: 251–256.
14. Muller C, Bang IS, Velayudhan J, Karlinsey J, Papenfort K, *et al.* (2009) Acid stress activation of the sigma(E) stress response in *Salmonella enterica* serovar Typhimurium. Mol Microbiol 71: 1228–1238.
15. Galan JE (1996) Molecular genetic bases of *Salmonella* entry into host cells. Mol Microbiol 20: 263–271.
16. Patel JC, Galan JE (2005) Manipulation of the host actin cytoskeleton by *Salmonella*--all in the name of entry. Curr Opin Microbiol 8: 10–15.
17. Jones BD, Ghori N, Falkow S (1994) *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. J Exp Med 180: 15–23.
18. Kohler H, Sakaguchi T, Hurley BP, Kase BA, Reinecker HC, *et al.* (2007) *Salmonella enterica* serovar Typhimurium regulates intercellular junction proteins and facilitates transepithelial neutrophil and bacterial passage. Am J Physiol Gastrointest Liver Physiol 293: G178–187.
19. Stecher B, Robbiani R, Walker AW, Westendorf AM, Barthel M, *et al.* (2007) *Salmonella enterica* serovar typhimurium exploits inflammation to compete with the intestinal microbiota. PLoS Biol 5: 2177–2189.
20. Raffatellu M, Chessa D, Wilson RP, Dusold R, Rubino S, *et al.* (2005) The Vi capsular antigen of *Salmonella enterica* serotype Typhi reduces Toll-like receptor-dependent interleukin-8 expression in the intestinal mucosa. Infect Immun 73: 3367–3374.

21. Sharma A, Qadri A (2004) Vi polysaccharide of *Salmonella typhi* targets the prohibitin family of molecules in intestinal epithelial cells and suppresses early inflammatory responses. Proc Natl Acad Sci U S A 101: 17492–17497.
22. Ames WR, Robins M (1943) Age and Sex as Factors in the Development of the Typhoid Carrier State, and a Method for Estimating Carrier Prevalence. Am J Public Health Nations Health 33: 221–230.
23. Crump JA, Luby SP, Mintz ED (2004) The global burden of typhoid fever. Bull World Health Organ 82: 346–353.
24. Saha SK, Baqui AH, Hanif M, Darmstadt GL, Ruhulamin M, *et al.* (2001) Typhoid fever in Bangladesh: implications for vaccination policy. Pediatr Infect Dis J 20: 521–524.
25. Ochiai RL, Acosta CJ, Danovaro-Holliday MC, Baiqing D, Bhattacharya SK, *et al.* (2008) A study of typhoid fever in five Asian countries: disease burden and implications for controls. Bull World Health Organ 86: 260–268.
26. Gilman RH, Terminel M, Levine MM, Hernandez-Mendoza P, Hornick RB (1975) Relative efficacy of blood, urine, rectal swab, bone-marrow, and rose-spot cultures for recovery of *Salmonella typhi* in typhoid fever. Lancet 1: 1211–1213.
27. Levine MM (2008) Typhoid fever vaccines. In: Plotkin SA, Orenstein WA, Offit PA, editors. Vaccines: Saunders. pp. 887–915.
28. Dupont HL, Hornick RB, Snyder MJ, Dawkins AT, Heiner GG, *et al.* (1971) Studies of immunity in typhoid fever. Protection induced by killed oral antigens or by primary infection. Bull World Health Organ 44: 667–672.
29. Woodward TE, Smadel JE, *et al.* (1948) Preliminary report on the beneficial effect of chloromycetin in the treatment of typhoid fever. Ann Intern Med 29: 131–134.
30. Mirza SH, Beeching NJ, Hart CA (1996) Multi-drug resistant typhoid: a global problem. J Med Microbiol 44: 317–319.
31. DuPont HL (1993) Quinolones in *Salmonella typhi* infection. Drugs 45 Suppl 3: 119–124.
32. Parry CM, Hien TT, Dougan G, White NJ, Farrar JJ (2002) Typhoid fever. N Engl J Med 347: 1770–1782.

33. Pfeiffer R, Kolle W (1896) Experimentelle Untersuchungen zur Frage der Schutzimpfung des Menschen gegen Typhus abdominalis. Dtsch Med Wocheschr 22: 735–737.
34. Wright A (1896) On the association of serous hemorrhages with conditions and defective blood coagulability. Lancet 2: 807–809.
35. Szu SC, Li XR, Stone AL, Robbins JB (1991) Relation between structure and immunologic properties of the Vi capsular polysaccharide. Infect Immun 59: 4555–4561.
36. Szu SC, Bystricky S (2003) Physical, chemical, antigenic, and immunologic characterization of polygalacturonan, its derivatives, and Vi antigen from *Salmonella typhi*. Methods Enzymol 363: 552–567.
37. Acharya IL, Lowe CU, Thapa R, Gurubacharya VL, Shrestha MB, *et al.* (1987) Prevention of typhoid fever in Nepal with the Vi capsular polysaccharide of *Salmonella typhi*. A preliminary report. N Engl J Med 317: 1101–1104.
38. Klugman KP, Gilbertson IT, Koornhof HJ, Robbins JB, Schneerson R, *et al.* (1987) Protective activity of Vi capsular polysaccharide vaccine against typhoid fever. Lancet 2: 1165–1169.
39. Plotkin SA, Bouveret-Le Cam N (1995) A new typhoid vaccine composed of the Vi capsular polysaccharide. Arch Intern Med 155: 2293–2299.
40. Keitel WA, Bond NL, Zahradnik JM, Cramton TA, Robbins JB (1994) Clinical and serological responses following primary and booster immunization with *Salmonella typhi* Vi capsular polysaccharide vaccines. Vaccine 12: 195–199.
41. Szu SC, Taylor DN, Trofa AC, Clements JD, Shiloach J, *et al.* (1994) Laboratory and preliminary clinical characterization of Vi capsular polysaccharide-protein conjugate vaccines. Infect Immun 62: 4440–4444.
42. Mirza NB, Wamola IA, Estambale BA, Mbithi E, Poillet M (1995) Typhim Vi vaccine against typhoid fever: a clinical trial in Kenya. East Afr Med J 72: 162–164.

43. Yang HH, Wu CG, Xie GZ, Gu QW, Wang BR, *et al.* (2001) Efficacy trial of Vi polysaccharide vaccine against typhoid fever in south-western China. Bull World Health Organ 79: 625–631.
44. Klugman KP, Koornhof HJ, Robbins JB, Le Cam NN (1996) Immunogenicity, efficacy and serological correlate of protection of *Salmonella typhi* Vi capsular polysaccharide vaccine three years after immunization. Vaccine 14: 435–438.
45. Pickard D, Wain J, Baker S, Line A, Chohan S, *et al.* (2003) Composition, acquisition, and distribution of the Vi exopolysaccharide-encoding *Salmonella enterica* pathogenicity island SPI-7. J Bacteriol 185: 5055–5065.
46. Bueno SM, Santiviago CA, Murillo AA, Fuentes JA, Trombert AN, *et al.* (2004) Precise excision of the large pathogenicity island, SPI7, in *Salmonella enterica* serovar Typhi. J Bacteriol 186: 3202–3213.
47. Hone DM, Attridge SR, Forrest B, Morona R, Daniels D, *et al.* (1988) A *galE* via (Vi antigen-negative) mutant of *Salmonella typhi* Ty2 retains virulence in humans. Infect Immun 56: 1326–1333.
48. Hornick RB, Greisman SE, Woodward TE, DuPont HL, Dawkins AT, *et al.* (1970) Typhoid fever: pathogenesis and immunologic control. N Engl J Med 283: 686–691.
49. Arya SC (2002) Field effectiveness of Vi polysaccharide typhoid vaccine in the People's Republic of China. J Infect Dis 185: 845; author reply 845–846.
50. Begier EM, Burwen DR, Haber P, Ball R, Vaccine Adverse Event Reporting System Working G (2004) Postmarketing safety surveillance for typhoid fever vaccines from the Vaccine Adverse Event Reporting System, July 1990 through June 2002. Clin Infect Dis 38: 771–779.
51. Germanier R, Fuer E (1975) Isolation and characterization of GalE mutant Ty21a of *Salmonella typhi*: a candidate strain for a live, oral typhoid vaccine. J Infect Dis 131: 553–558.
52. Hone D, Morona R, Attridge S, Hackett J (1987) Construction of defined *galE* mutants of *Salmonella* for use as vaccines. J Infect Dis 156: 167–174.

53. Gilman RH, Hornick RB, Woodard WE, DuPont HL, Snyder MJ, *et al.* (1977) Evaluation of a UDP-glucose-4-epimerase-less mutant of *Salmonella typhi* as a liver oral vaccine. J Infect Dis 136: 717–723.
54. Kopecko DJ, Sieber H, Ures JA, Furer A, Schlup J, *et al.* (2009) Genetic stability of vaccine strain *Salmonella* Typhi Ty21a over 25 years. Int J Med Microbiol 299: 233–246.
55. Robbe-Saule V, Coynault C, Norel F (1995) The live oral typhoid vaccine Ty21a is a *rpoS* mutant and is susceptible to various environmental stresses. FEMS Microbiol Lett 126: 171–176.
56. Levine MM, Ferreccio C, Black RE, Tacket CO, Germanier R (1989) Progress in vaccines against typhoid fever. Rev Infect Dis 11 Suppl 3: S552–567.
57. Levine MM, Ferreccio C, Black RE, Germanier R (1987) Large-scale field trial of Ty21a live oral typhoid vaccine in enteric-coated capsule formulation. Lancet 1: 1049–1052.
58. Black RE, Levine MM, Ferreccio C, Clements ML, Lanata C, *et al.* (1990) Efficacy of one or two doses of Ty21a *Salmonella typhi* vaccine in enteric-coated capsules in a controlled field trial. Chilean Typhoid Committee. Vaccine 8: 81–84.
59. Ferreccio C, Levine MM, Rodriguez H, Contreras R (1989) Comparative efficacy of two, three, or four doses of TY21a live oral typhoid vaccine in enteric-coated capsules: a field trial in an endemic area. J Infect Dis 159: 766–769.
60. Hone DM, Harris AM, Chatfield S, Dougan G, Levine MM (1991) Construction of genetically defined double *aro* mutants of *Salmonella typhi*. Vaccine 9: 810–816.
61. Levine MM, Galen J, Barry E, Noriega F, Chatfield S, *et al.* (1996) Attenuated *Salmonella* as live oral vaccines against typhoid fever and as live vectors. J Biotechnol 44: 193–196.
62. Chatfield SN, Strahan K, Pickard D, Charles IG, Hormaeche CE, *et al.* (1992) Evaluation of *Salmonella typhimurium* strains harbouring defined mutations in *htrA* and *aroA* in the murine salmonellosis model. Microb Pathog 12: 145–151.

63. Tacket CO, Sztein MB, Losonsky GA, Wasserman SS, Nataro JP, *et al.* (1997) Safety of live oral *Salmonella typhi* vaccine strains with deletions in *htrA* and *aroC aroD* and immune response in humans. Infect Immun 65: 452–456.
64. Tacket CO, Sztein MB, Wasserman SS, Losonsky G, Kotloff KL, *et al.* (2000) Phase 2 clinical trial of attenuated *Salmonella enterica* serovar typhi oral live vector vaccine CVD 908-*htrA* in U.S. volunteers. Infect Immun 68: 1196–1201.
65. Salerno-Goncalves R, Wyant TL, Pasetti MF, Fernandez-Vina M, Tacket CO, *et al.* (2003) Concomitant induction of CD4+ and CD8+ T cell responses in volunteers immunized with Salmonella enterica serovar typhi strain CVD 908-*htrA*. J Immunol 170: 2734–2741.
66. Arricau N, Hermant D, Waxin H, Ecobichon C, Duffey PS, *et al.* (1998) The RcsB-RcsC regulatory system of *Salmonella typhi* differentially modulates the expression of invasion proteins, flagellin and Vi antigen in response to osmolarity. Mol Microbiol 29: 835–850.
67. Wang JY, Noriega FR, Galen JE, Barry E, Levine MM (2000) Constitutive expression of the Vi polysaccharide capsular antigen in attenuated *Salmonella enterica* serovar typhi oral vaccine strain CVD 909. Infect Immun 68: 4647–4652.
68. Tacket CO, Pasetti MF, Sztein MB, Livio S, Levine MM (2004) Immune responses to an oral typhoid vaccine strain that is modified to constitutively express Vi capsular polysaccharide. J Infect Dis 190: 565–570.
69. Hohmann EL, Oletta CA, Killeen KP, Miller SI (1996) *phoP*/*phoQ*-deleted *Salmonella typhi* (Ty800) is a safe and immunogenic single-dose typhoid fever vaccine in volunteers. J Infect Dis 173: 1408–1414.
70. Hindle Z, Chatfield SN, Phillimore J, Bentley M, Johnson J, et al. (2002) Characterization of *Salmonella enterica* derivatives harboring defined *aroC* and *Salmonella* pathogenicity island 2 type III secretion system (*ssaV*) mutations by immunization of healthy volunteers. Infect Immun 70: 3457–3467.

71. Kirkpatrick BD, Tenney KM, Larsson CJ, O'Neill JP, Ventrone C, *et al.* (2005) The novel oral typhoid vaccine M01ZH09 is well tolerated and highly immunogenic in 2 vaccine presentations. J Infect Dis 192: 360–366.
72. Claesson BA, Schneerson R, Trollfors B, Lagergard T, Taranger J, *et al.* (1990) Duration of serum antibodies elicited by *Haemophilus influenzae* type b capsular polysaccharide alone or conjugated to tetanus toxoid in 18- to 23-month-old children. J Pediatr 116: 929–931.
73. Bulkow LR, Wainwright RB, Letson GW, Chang SJ, Ward JI (1993) Comparative immunogenicity of four *Haemophilus influenzae* type b conjugate vaccines in Alaska Native infants. Pediatr Infect Dis J 12: 484–492.
74. Parijs BA, Malinoski FJ (2004) Post-marketing effectiveness of Prevnar [pneumococcal 7-valent conjugate vaccine (diphtheria CRM197 protein)] and implications for adult immunization. Mech Ageing Dev 125: 147–148.
75. Riddell A, Buttery JP, McVernon J, Chantler T, Lane L, *et al.* (2007) A randomized study comparing the safety and immunogenicity of a conjugate vaccine combination containing meningococcal group C and pneumococcal capsular polysaccharide--CRM197 with a meningococcal group C conjugate vaccine in healthy infants: challenge phase. Vaccine 25: 3906–3912.
76. Safadi MA, Barros AP (2006) Meningococcal conjugate vaccines: efficacy and new combinations. J Pediatr (Rio J) 82: S35–44.
77. Kossaczka Z, Bystricky S, Bryla DA, Shiloach J, Robbins JB, *et al.* (1997) Synthesis and immunological properties of Vi and di-O-acetyl pectin protein conjugates with adipic acid dihydrazide as the linker. Infect Immun 65: 2088–2093.
78. Szu SC, Li XR, Schneerson R, Vickers JH, Bryla D, *et al.* (1989) Comparative immunogenicities of Vi polysaccharide-protein conjugates composed of cholera toxin or its B subunit as a carrier bound to high- or lower-molecular-weight Vi. Infect Immun 57: 3823–3827.

79. Kossaczka Z, Lin FY, Ho VA, Thuy NT, Van Bay P, *et al.* (1999) Safety and immunogenicity of Vi conjugate vaccines for typhoid fever in adults, teenagers, and 2- to 4-year-old children in Vietnam. Infect Immun 67: 5806–5810.
80. Lin FY, Ho VA, Khiem HB, Trach DD, Bay PV, *et al.* (2001) The efficacy of a *Salmonella typhi* Vi conjugate vaccine in two-to-five-year-old children. N Engl J Med 344: 1263–1269.
81. Mai NL, Phan VB, Vo AH, Tran CT, Lin FY, *et al.* (2003) Persistent efficacy of Vi conjugate vaccine against typhoid fever in young children. N Engl J Med 349: 1390–1391.
82. Canh DG, Lin FY, Thiem VD, Trach DD, Trong ND, *et al.* (2004) Effect of dosage on immunogenicity of a Vi conjugate vaccine injected twice into 2- to 5-year-old Vietnamese children. Infect Immun 72: 6586–6588.
83. Thiem VD, Lin FY, Canh do G, Son NH, Anh DD, *et al.* (2011) The Vi conjugate typhoid vaccine is safe, elicits protective levels of IgG anti-Vi, and is compatible with routine infant vaccines. Clin Vaccine Immunol 18: 730–735.
84. McClelland M, Sanderson KE, Clifton SW, Latreille P, Porwollik S, *et al.* (2004) Comparison of genome degradation in Paratyphi A and Typhi, human-restricted serovars of *Salmonella enterica* that cause typhoid. Nat Genet 36: 1268–1274.
85. Holt KE, Thomson NR, Wain J, Langridge GC, Hasan R, *et al.* (2009) Pseudogene accumulation in the evolutionary histories of *Salmonella enterica* serovars Paratyphi A and Typhi. BMC Genomics 10: 36.
86. Levine MM, Ferreccio C, Black RE, Lagos R, San Martin O, *et al.* (2007) Ty21a live oral typhoid vaccine and prevention of paratyphoid fever caused by *Salmonella enterica* Serovar Paratyphi B. Clin Infect Dis 45 Suppl 1: S24–28.
87. Meltzer E, Sadik C, Schwartz E (2005) Enteric fever in Israeli travelers: a nationwide study. J Travel Med 12: 275–281.
88. Namgyal P (2010) Feedback from the regions and countries on the implementation of SAGE recommendations on typhoid vaccines. WHO.

89. Dong BQ, Yang J, Wang XY, Gong J, von Seidlein L, *et al.* (2010) Trends and disease burden of enteric fever in Guangxi province, China, 1994–2004. Bull World Health Organ 88: 689–696.
90. Woods CW, Murdoch DR, Zimmerman MD, Glover WA, Basnyat B, *et al.* (2006) Emergence of *Salmonella enterica* serotype Paratyphi A as a major cause of enteric fever in Kathmandu, Nepal. Trans R Soc Trop Med Hyg 100: 1063–1067.
91. Ochiai RL, Wang X, von Seidlein L, Yang J, Bhutta ZA, *et al.* (2005) *Salmonella paratyphi A* rates, Asia. Emerg Infect Dis 11: 1764–1766.
92. Konadu EY, Lin FY, Ho VA, Thuy NT, Van Bay P, *et al.* (2000) Phase 1 and phase 2 studies of *Salmonella enterica* serovar paratyphi A O-specific polysaccharide-tetanus toxoid conjugates in adults, teenagers, and 2- to 4-year-old children in Vietnam. Infect Immun 68: 1529–1534.
93. Konadu E, Shiloach J, Bryla DA, Robbins JB, Szu SC (1996) Synthesis, characterization, and immunological properties in mice of conjugates composed of detoxified lipopolysaccharide of *Salmonella paratyphi A* bound to tetanus toxoid with emphasis on the role of O acetyls. Infect Immun 64: 2709–2715.
94. Hendriksen RS, Vieira AR, Karlsmose S, Lo Fo Wong DM, Jensen AB, *et al.* (2011) Global monitoring of *Salmonella* serovar distribution from the World Health Organization Global Foodborne Infections Network Country Data Bank: results of quality assured laboratories from 2001 to 2007. Foodborne Pathog Dis 8: 887–900.
95. Gordon MA, Graham SM, Walsh AL, Wilson L, Phiri A, *et al.* (2008) Epidemics of invasive *Salmonella enterica* serovar enteritidis and *S. enterica* serovar typhimurium infection associated with multidrug resistance among adults and children in Malawi. Clin Infect Dis 46: 963–969.
96. Svenson SB, Lindberg AA (1981) Artificial *Salmonella* vaccines: *Salmonella typhimurium* O-antigen-specific oligosaccharide-protein conjugates elicit protective antibodies in rabbits and mice. Infect Immun 32: 490–496.

97. Watson DC, Robbins JB, Szu SC (1992) Protection of mice against *Salmonella typhimurium* with an O-specific polysaccharide-protein conjugate vaccine. Infect Immun 60: 4679–4686.
98. MacLennan CA, Gilchrist JJ, Gordon MA, Cunningham AF, Cobbold M, *et al.* (2010) Dysregulated humoral immunity to nontyphoidal *Salmonella* in HIV-infected African adults. Science 328: 508–512.
99. Lee SJ, Liang L, Juarez S, Nanton MR, Gondwe EN, *et al.* (2012) Identification of a common immune signature in murine and human systemic Salmonellosis. Proc Natl Acad Sci U S A.
100. Young NM, Brisson JR, Kelly J, Watson DC, Tessier L, *et al.* (2002) Structure of the N-linked glycan present on multiple glycoproteins in the Gram-negative bacterium, *Campylobacter jejuni*. J Biol Chem 277: 42530–42539.
101. Wacker M, Linton D, Hitchen PG, Nita-Lazar M, Haslam SM, *et al.* (2002) N-linked glycosylation in *Campylobacter jejuni* and its functional transfer into *E. coli*. Science 298: 1790–1793.
102. Feldman MF, Wacker M, Hernandez M, Hitchen PG, Marolda CL, *et al.* (2005) Engineering N-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in *Escherichia coli*. Proc Natl Acad Sci U S A 102: 3016–3021.
103. Wacker M, Feldman MF, Callewaert N, Kowarik M, Clarke BR, *et al.* (2006) Substrate specificity of bacterial oligosaccharyltransferase suggests a common transfer mechanism for the bacterial and eukaryotic systems. Proc Natl Acad Sci U S A 103: 7088–7093.
104. Kowarik M, Young NM, Numao S, Schulz BL, Hug I, *et al.* (2006) Definition of the bacterial N-glycosylation site consensus sequence. EMBO J 25: 1957–1966.
105. Ihssen J, Kowarik M, Dilettoso S, Tanner C, Wacker M, *et al.* (2010) Production of glycoprotein vaccines in *Escherichia coli*. Microb Cell Fact 9: 61.
106. Whitfield C (2006) Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. Annu Rev Biochem 75: 39–68.

107. Hashimoto Y, Ezaki T, Li N, Yamamoto H (1991) Molecular cloning of the ViaB region of *Salmonella typhi*. FEMS Microbiol Lett 69: 53–56.
108. Bogomolnaya LM, Santiviago CA, Yang HJ, Baumler AJ, Andrews-Polymenis HL (2008) 'Form variation' of the O12 antigen is critical for persistence of *Salmonella* Typhimurium in the murine intestine. Mol Microbiol 70: 1105–1119.
109. Slauch JM, Mahan MJ, Michetti P, Neutra MR, Mekalanos JJ (1995) Acetylation (O-factor 5) affects the structural and immunological properties of *Salmonella typhimurium* lipopolysaccharide O antigen. Infect Immun 63: 437–441.
110. Raetz CR, Whitfield C (2002) Lipopolysaccharide endotoxins. Annu Rev Biochem 71: 635–700.
111. Slauch JM, Lee AA, Mahan MJ, Mekalanos JJ (1996) Molecular characterization of the *oafA* locus responsible for acetylation of *Salmonella typhimurium* O-antigen: *oafA* is a member of a family of integral membrane trans-acylases. J Bacteriol 178: 5904–5909.
112. Allison GE, Verma NK (2000) Serotype-converting bacteriophages and O-antigen modification in *Shigella flexneri*. Trends Microbiol 8: 17–23.
113. Nikaido H, Nikaido K, Nakae T, Makela PH (1971) Glucosylation of lipopolysaccharide in *Salmonella*: biosynthesis of O antigen factor 12 2 . I. Over-all reaction. J Biol Chem 246: 3902–3911.
114. Nikaido K, Nikaido H (1971) Glucosylation of lipopolysaccharide in *Salmonella*: biosynthesis nof O antigen factor n12 2 . II. Structure of the lipid intermediate. J Biol Chem 246: 3912–3919.
115. Verma N, Reeves P (1989) Identification and sequence of *rfbS* and *rfbE*, which determine antigenic specificity of group A and group D salmonellae. J Bacteriol 171: 5694–5701.
116. Looney RJ, Steigbigel RT (1986) Role of the Vi antigen of *Salmonella typhi* in resistance to host defense in vitro. J Lab Clin Med 108: 506–516.

117. Robbins JD, Robbins JB (1984) Reexamination of the protective role of the capsular polysaccharide (Vi antigen) of *Salmonella typhi*. J Infect Dis 150: 436–449.
118. Engels EA, Falagas ME, Lau J, Bennish ML (1998) Typhoid fever vaccines: a meta-analysis of studies on efficacy and toxicity. Bmj 316: 110–116.
119. Houng HS, Noon KF, Ou JT, Baron LS (1992) Expression of Vi antigen in *Escherichia coli* K-12: characterization of ViaB from *Citrobacter freundii* and identity of ViaA with RcsB. J Bacteriol 174: 5910–5915.
120. Virlogeux I, Waxin H, Ecobichon C, Lee JO, Popoff MY (1996) Characterization of the *rcsA* and *rcsB* genes from *Salmonella typhi*: *rcsB* through *tviA* is involved in regulation of Vi antigen synthesis. J Bacteriol 178: 1691–1698.
121. Pickard D, Li J, Roberts M, Maskell D, Hone D, *et al.* (1994) Characterization of defined *ompR* mutants of *Salmonella typhi*: *ompR* is involved in the regulation of Vi polysaccharide expression. Infect Immun 62: 3984–3993.
122. Hashimoto Y, Li N, Yokoyama H, Ezaki T (1993) Complete nucleotide sequence and molecular characterization of ViaB region encoding Vi antigen in *Salmonella typhi*. J Bacteriol 175: 4456–4465.
123. Virlogeux I, Waxin H, Ecobichon C, Popoff MY (1995) Role of the *viaB* locus in synthesis, transport and expression of *Salmonella typhi* Vi antigen. Microbiology 141 (Pt 12): 3039–3047.
124. Zhang H, Zhou Y, Bao H, Liu HW (2006) Vi antigen biosynthesis in *Salmonella typhi*: characterization of UDP-N-acetylglucosamine C-6 dehydrogenase (TviB) and UDP-N-acetylglucosaminuronic acid C-4 epimerase (TviC). Biochemistry 45: 8163–8173.
125. Cuthbertson L, Mainprize IL, Naismith JH, Whitfield C (2009) Pivotal roles of the outer membrane polysaccharide export and polysaccharide copolymerase protein families in export of extracellular polysaccharides in gram-negative bacteria. Microbiol Mol Biol Rev 73: 155–177.

126. Dong C, Beis K, Nesper J, Brunkan-Lamontagne AL, Clarke BR, *et al.* (2006) Wza the translocon for *E. coli* capsular polysaccharides defines a new class of membrane protein. Nature 444: 226–229.
127. Sathiyamoorthy K, Mills E, Franzmann TM, Rosenshine I, Saper MA (2011) The crystal structure of *Escherichia coli* group 4 capsule protein GfcC reveals a domain organization resembling that of Wza. Biochemistry 50: 5465–5476.
128. Bliss JM, Silver RP (1996) Coating the surface: a model for expression of capsular polysialic acid in *Escherichia coli* K1. Mol Microbiol 21: 221–231.
129. Craigie J, Brandon KF (1936) Bacteriophage specific for the O-resistant V form of *B. typhosus*. J Pathol Bacteriol 43: 233–248.
130. Craigie J, Yen CH (1937) V bacteriophages for *B. typhosus*. Trans R Soc Can 5: 79–87.
131. Craigie J, Yen CH (1938) The demonstration of types of *B. typhosus* by means of preparations of type II Vi phage. I. Principles and technique. Can Public Health J 29: 448–463.
132. Desranleau JM (1949) Progress in the treatment of typhoid fever with Vi bacteriophages. Can J Public Health 40: 473–478.
133. Brandis H, Imamura PS (1956) [Comparative studies of Vi-bacteriophages]. Z Hyg Infektionskr 143: 50–61.
134. Qadri A, Ghosh S, Talwar GP (1990) Monoclonal antibodies against two discrete determinants on Vi capsular polysaccharide. J Immunoassay 11: 235–250.
135. Taylor K (1965) Enzymatic deacetylation of Vi-polysaccharide by Vi-phage. II. Biochem Biophys Res Commun 20: 752–756.
136. Hestrin S (1949) The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine, and its analytical application. J Biol Chem 180: 249–261.
137. Bergfeld AK, Claus H, Vogel U, Muhlenhoff M (2007) Biochemical characterization of the polysialic acid-specific O-acetyltransferase NeuO of *Escherichia coli* K1. J Biol Chem 282: 22217–22227.

138. Johnson EM, Baron LS (1969) Genetic transfer of the Vi antigen from *Salmonella typhosa* to *Escherichia coli*. J Bacteriol 99: 358–359.
139. Pickard D, Toribio AL, Petty NK, van Tonder A, Yu L, *et al.* A conserved acetyl esterase domain targets diverse bacteriophages to the Vi capsular receptor of *Salmonella enterica* serovar Typhi. J Bacteriol 192: 5746–5754.
140. Friedman AM, Long SR, Brown SE, Buikema WJ, Ausubel FM (1982) Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene 18: 289–296.
141. Nair S, Alokam S, Kothapalli S, Porwollik S, Proctor E, *et al.* (2004) *Salmonella enterica* serovar Typhi strains from which SPI7, a 134-kilobase island with genes for Vi exopolysaccharide and other functions, has been deleted. J Bacteriol 186: 3214–3223.
142. Annunziato PW, Wright LF, Vann WF, Silver RP (1995) Nucleotide sequence and genetic analysis of the *neuD* and *neuB* genes in region 2 of the polysialic acid gene cluster of *Escherichia coli* K1. J Bacteriol 177: 312–319.
143. Troy FA, 2nd (1992) Polysialylation: from bacteria to brains. Glycobiology 2: 5–23.
144. Pavelka MS, Jr., Hayes SF, Silver RP (1994) Characterization of KpsT, the ATP-binding component of the ABC-transporter involved with the export of capsular polysialic acid in *Escherichia coli* K1. J Biol Chem 269: 20149–20158.
145. Rigg GP, Barrett B, Roberts IS (1998) The localization of KpsC, S and T, and KfiA, C and D proteins involved in the biosynthesis of the *Escherichia coli* K5 capsular polysaccharide: evidence for a membrane-bound complex. Microbiology 144 (Pt 10): 2905–2914.
146. Steenbergen SM, Vimr ER (2008) Biosynthesis of the *Escherichia coli* K1 group 2 polysialic acid capsule occurs within a protected cytoplasmic compartment. Mol Microbiol 68: 1252–1267.

147. Bronner D, Sieberth V, Pazzani C, Roberts IS, Boulnois GJ, *et al.* (1993) Expression of the capsular K5 polysaccharide of *Escherichia coli*: biochemical and electron microscopic analyses of mutants with defects in region 1 of the K5 gene cluster. J Bacteriol 175: 5984–5992.
148. Wunder DE, Aaronson W, Hayes SF, Bliss JM, Silver RP (1994) Nucleotide sequence and mutational analysis of the gene encoding KpsD, a periplasmic protein involved in transport of polysialic acid in *Escherichia coli* K1. J Bacteriol 176: 4025–4033.
149. Bliss JM, Garon CF, Silver RP (1996) Polysialic acid export in *Escherichia coli* K1: the role of KpsT, the ATP-binding component of an ABC transporter, in chain translocation. Glycobiology 6: 445–452.
150. Demil P, D'Hondt E, Van Hoecke C (2003) *Salmonella* Typhi vaccine compositions. In: S.A. GB, editor. EP 1 107 787 B1.
151. Jansen AM, Hall LJ, Clare S, Goulding D, Holt KE, *et al.* A *Salmonella* Typhimurium-Typhi genomic chimera: a model to study Vi polysaccharide capsule function *in vivo*. PLoS Pathog 7: e1002131.
152. Bhan MK, Bahl R, Bhatnagar S (2005) Typhoid and paratyphoid fever. Lancet 366: 749–762.
153. Murphy JR, Grez L, Schlesinger L, Ferreccio C, Baqar S, *et al.* (1991) Immunogenicity of *Salmonella typhi* Ty21a vaccine for young children. Infect Immun 59: 4291–4293.
154. Levine MM, Ferreccio C, Cryz S, Ortiz E (1990) Comparison of enteric-coated capsules and liquid formulation of Ty21a typhoid vaccine in randomised controlled field trial. Lancet 336: 891–894.
155. Levine MM, Ferreccio C, Abrego P, Martin OS, Ortiz E, *et al.* (1999) Duration of efficacy of Ty21a, attenuated *Salmonella typhi* live oral vaccine. Vaccine 17 Suppl 2: S22–27.
156. Weintraub A (2003) Immunology of bacterial polysaccharide antigens. Carbohydr Res 338: 2539–2547.
157. Landy M (1954) Studies on Vi antigen. VI. Immunization of human beings with purified Vi antigen. Am J Hyg 60: 52–62.

158. Parolis H, Parolis LA, Olivieri G (1997) Structural studies on the *Shigella*-like *Escherichia coli* O121 O-specific polysaccharide. Carbohydr Res 303: 319–325.
159. King JD, Vinogradov E, Tran V, Lam JS (2010) Biosynthesis of uronamide sugars in *Pseudomonas aeruginosa* O6 and *Escherichia coli* O121 O antigens. Environ Microbiol 12: 1531–1544.
160. Liu D, Reeves PR (1994) *Escherichia coli* K12 regains its O antigen. Microbiology 140 (Pt 1): 49–57.
161. Feldman MF, Marolda CL, Monteiro MA, Perry MB, Parodi AJ, *et al.* (1999) The activity of a putative polyisoprenol-linked sugar translocase (Wzx) involved in *Escherichia coli* O antigen assembly is independent of the chemical structure of the O repeat. J Biol Chem 274: 35129–35138.
162. Kubler-Kielb J, Coxon B, Schneerson R (2004) Chemical structure, conjugation, and cross-reactivity of *Bacillus pumilus* Sh18 cell wall polysaccharide. J Bacteriol 186: 6891–6901.
163. Kubler-Kielb J, Vinogradov E, Hu H, Leppla SH, Robbins JB, *et al.* (2008) Saccharides cross-reactive with *Bacillus anthracis* spore glycoprotein as an anthrax vaccine component. Proc Natl Acad Sci U S A 105: 8709–8712.
164. Laemmli UK, Favre M (1973) Maturation of the head of bacteriophage T4. I. DNA packaging events. J Mol Biol 80: 575–599.
165. Tsai CM, Frasch CE (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal Biochem 119: 115–119.
166. Glover KJ, Weerapana E, Imperiali B (2005) *In vitro* assembly of the undecaprenylpyrophosphate-linked heptasaccharide for prokaryotic N-linked glycosylation. Proc Natl Acad Sci U S A 102: 14255–14259.
167. Bigge JC, Patel TP, Bruce JA, Goulding PN, Charles SM, *et al.* (1995) Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid. Anal Biochem 230: 229–238.

168. Merry AH, Neville DC, Royle L, Matthews B, Harvey DJ, *et al.* (2002) Recovery of intact 2-aminobenzamide-labeled O-glycans released from glycoproteins by hydrazinolysis. Anal Biochem 304: 91–99.
169. Royle L, Mattu TS, Hart E, Langridge JI, Merry AH, *et al.* (2002) An analytical and structural database provides a strategy for sequencing O-glycans from microgram quantities of glycoproteins. Anal Biochem 304: 70–90.
170. Apicella MA (2008) Isolation and characterization of lipopolysaccharides. Methods Mol Biol 431: 3–13.
171. Frey A, Di Canzio J, Zurakowski D (1998) A statistically defined endpoint titer determination method for immunoassays. J Immunol Methods 221: 35–41.
172. Dykxhoorn DM, St Pierre R, Linn T (1996) A set of compatible *tac* promoter expression vectors. Gene 177: 133–136.
173. Helenius A, Aebi M (2004) Roles of N-linked glycans in the endoplasmic reticulum. Annu Rev Biochem 73: 1019–1049.
174. Kornfeld R, Kornfeld S (1985) Assembly of asparagine-linked oligosaccharides. Annu Rev Biochem 54: 631–664.
175. Varki A (1993) Biological roles of oligosaccharides: all of the theories are correct. Glycobiology 3: 97–130.
176. Kelleher DJ, Gilmore R (2006) An evolving view of the eukaryotic oligosaccharyltransferase. Glycobiology 16: 47R-62R.
177. Zufferey R, Knauer R, Burda P, Stagljar I, te Heesen S, *et al.* (1995) STT3, a highly conserved protein required for yeast oligosaccharyl transferase activity *in vivo*. Embo J 14: 4949–4960.
178. Yan Q, Lennarz WJ (2002) Studies on the function of oligosaccharyl transferase subunits: a glycosylatable photoprobe binds to the luminal domain of Ost1p. Proc Natl Acad Sci U S A 99: 15994–15999.
179. Langdon RH, Cuccui J, Wren BW (2009) N-linked glycosylation in bacteria: an unexpected application. Future Microbiol 4: 401–412.
180. Szymanski CM, Yao R, Ewing CP, Trust TJ, Guerry P (1999) Evidence for a system of general protein glycosylation in *Campylobacter jejuni*. Mol Microbiol 32: 1022–1030.

181. Glover KJ, Weerapana E, Chen MM, Imperiali B (2006) Direct biochemical evidence for the utilization of UDP-bacillosamine by PglC, an essential glycosyl-1-phosphate transferase in the *Campylobacter jejuni* N-linked glycosylation pathway. Biochemistry 45: 5343–5350.
182. Olivier NB, Chen MM, Behr JR, Imperiali B (2006) *In vitro* biosynthesis of UDP-N,N'-diacetylbacillosamine by enzymes of the *Campylobacter jejuni* general protein glycosylation system. Biochemistry 45: 13659–13669.
183. Linton D, Dorrell N, Hitchen PG, Amber S, Karlyshev AV, *et al.* (2005) Functional analysis of the *Campylobacter jejuni* N-linked protein glycosylation pathway. Mol Microbiol 55: 1695–1703.
184. Alaimo C, Catrein I, Morf L, Marolda CL, Callewaert N, *et al.* (2006) Two distinct but interchangeable mechanisms for flipping of lipid-linked oligosaccharides. Embo J 25: 967–976.
185. Kowarik M, Numao S, Feldman MF, Schulz BL, Callewaert N, *et al.* (2006) N-linked glycosylation of folded proteins by the bacterial oligosaccharyltransferase. Science 314: 1148–1150.
186. Lizak C, Gerber S, Numao S, Aebi M, Locher KP (2011) X-ray structure of a bacterial oligosaccharyltransferase. Nature 474: 350–355.
187. Chen MM, Weerapana E, Ciepichal E, Stupak J, Reid CW, *et al.* (2007) Polyisoprenol specificity in the *Campylobacter jejuni* N-linked glycosylation pathway. Biochemistry 46: 14342–14348.
188. Murray GL, Attridge SR, Morona R (2003) Regulation of *Salmonella typhimurium* lipopolysaccharide O antigen chain length is required for virulence; identification of FepE as a second Wzz. Mol Microbiol 47: 1395–1406.
189. Wang L, Liu D, Reeves PR (1996) C-terminal half of *Salmonella enterica* WbaP (RfbP) is the galactosyl-1-phosphate transferase domain catalyzing the first step of O-antigen synthesis. J Bacteriol 178: 2598–2604.
190. Whitney CG, Farley MM, Hadler J, Harrison LH, Bennett NM, *et al.* (2003) Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. N Engl J Med 348: 1737–1746.

191. Pai R, Moore MR, Pilishvili T, Gertz RE, Whitney CG, *et al.* (2005) Postvaccine genetic structure of *Streptococcus pneumoniae* serotype 19A from children in the United States. J Infect Dis 192: 1988–1995.
192. Bloor AE, Cranenburgh RM (2006) An efficient method of selectable marker gene excision by Xer recombination for gene replacement in bacterial chromosomes. Appl Environ Microbiol 72: 2520–2525.
193. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97: 6640–6645.

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